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Proteomic Analysis of Rat Prefrontal Cortex in Three Phases of Morphine-Induced Conditioned Place Preference

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Morphological alterations of synapse are found after morphine administration, suggesting that regulation of synaptic plasticity may be one of the mechanisms of neuroadaptation in addiction. However, the molecular basis underlying the abnormal synapse morphological and physiological changes in the morphine-induced dependence, withdraw, and relapse is not well understood. As prefrontal cortex (PFC) is one of the most important brain regions, which provides executive control over drug use and is severely impaired in many addicts, systematic analysis of the biochemical and molecular alteration of synaptic fraction of PFC in morphine-induced neuroadaptation is necessary. In this study, differential protein expression profiling of synaptic fraction of rat PFC based on morphine-induced conditioned place preference (CPP) model was performed with two-dimensional gel electrophoresis (2-DE). Our results showed that a total of 80 proteins were differentially expressed by 2-DE analysis during three phases of CPP assay. Of them, 58 were further identified by mass spectrometry. These proteins were classified into multiple categories, such as energy metabolism, signal transduction, synaptic transmission, cytoskeletal proteins, chaperones, and local synaptic protein synthetic machinery according to their biological functions. Our study provides a global view of synaptic-related molecular networking in PFC under morphine-induced dependence, withdraw, and relapse, indicative of a concerted biological process in neuroadaptation under chronic morphine exposure.

Keywords: morphine addiction • conditioned place preference (CPP) • prefrontal cortex (PFC) • proteomics • neuroplasticity

1. Introduction

Drug addiction has been recognized as a chronic and relapsing mental disorder involving the development of complex behaviors such as drug tolerance, dependence, and craving for the drugs that are characteristics of an addicted state. Repeated or intermittent morphine abuse can produce tolerance, sensitization, and dependence. It is known that morphine treatment may promote a widespread reorganization of synaptic connectivity in the forebrain.[1] Recent studies also demonstrated that morphine could induce alteration in dendritic structure[2,3] and markedly decreased the spine density in the nucleus accumbens (NAc) and medial prefrontal cortex (mPFC).[4]

Prefrontal cortex (PFC) is a brain region which plays a role in cognitive control and working memory and could connect and modulate comprehensive information to guide behavior. It has been hypothesized that PFC has the function of representation of goals, valuation, and selecting associated action based on the evaluation.[5] Drug taking can distort the representation of goals and induce abnormal behavior. Additionally, it has been shown that drug taking impairs the top-down control in behavior by producing pathological adaptations in PFC.[6] Neuroimaging studies in addicts also demonstrated that metabolic activation of PFC was associated with craving for cocaine,[7,8] suggesting PFC is one of major brain regions which is associated with drug addiction. Thus, it is suggested that PFC may play an important role in morphine-induced neuroplasticity, but the biochemical and molecular mechanisms responsible for the change in neuroplasticity have not been fully elucidated.

Conditioned place preference (CPP) is a well-established animal model to measure the rewarding or incentive properties of drugs.[9] It also can be served as a tool to measure the priming effect of a drug on relapse following extinction training.[10] A large number of studies have confirmed that morphine can induce CPP in rats.[11,12] As CPP procedure can be used to mimic the development of addiction state from acquisition, extinction to reinstatement, CPP is ideal for evaluating the effects of morphine in chronic addiction.

Many candidate molecules, such as ΔFosB, cJun, GluR2, Cdk5, CREB, cFos, calcium/calmodulin-dependent protein kinase type IV (CaMKIV), Homer, Narp (neuronal-activity-

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regulated pentraxin), and Arc (activity-regulated cytoskeleton-associated protein) have been found to contribute to the persistence of addiction.[13] However, all of these molecules were identified by traditional molecular and biochemical approaches, which are based on piece-by-piece or gene-by-gene methodologies, and may only represent a partial aspect of general mechanism for addiction. With the advancement in proteomics technology, it becomes possible to analyze protein expression profile on whole genome level for drug addiction. In the current study, we employed unbiased 2-DE-based analytical methodology in combination with matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF−MS) to explore the underlying synaptic-related biochemical and molecular alteration in PFC during three phases of CPP induced by morphine.

2. Materials and Methods

2.1. Animals. This study was approved by Animal Use Committee of Zhejiang University. Ninety-six male Spraque-Dowley rats weighing 180−220 g were supplied by Zhejiang Medical Science Research Institute. They were randomly divided into two groups (48 rats/group), saline-treatment group and morphine-treatment group. The rats were housed individually in polypropylene boxes and fed food and water ad libitum. All rats were allowed to be acclimated to the colony room for 2 weeks upon arrival, and maintained on a 12 h light−dark cycle (light on at 7:00 p.m.). Room temperature was maintained at (22 ± 2) °C.

2.2. Reagents and Materials. Morphine hydrochloric was supplied by Shenyang Pharmaceutical LTD, China. Trypsin (sequencing grade) and mouse monoclonal anti-β-actin were purchased from Sigma (St. Louis, MO). Dithiothreitol (DTT), iodoacetamide, urea, CHAPS, acrylamide, Bis-acrylamide, Tris-base, SDS, ammonium persulfate, and TEMED were supplied from Promega (San Luis Obispo, CA). IPG buffer (3−10 NL) and IPG Strips (3−10 NL) were provided from Amersham Pharmacia (Piscataway, NJ). Goat anti-CNPase1 polyclonal antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). IRDyeTM800CW conjugated affinity purified anti-Goat IgG was from Rockland (Gilbertsville, PA).

2.3. Animal Model and Sample Preparation. We used CPP model to evaluate morphine-induced addiction. The computer-based video-tracking CPP system consists of three parts, including CPP training apparatus, a video camera, and a computer system with analysis software. The CPP training apparatus and protocol were based on the approach described from Shippenberg et al.[14] with some modification. Briefly, the procedure included acquisition of CPP (consisting of three phases: pre-exposure, conditioning training, and conditioning test), extinction training, and reinstatement.[15] Each animal received a pre-exposure test for 3 days to access to the entire apparatus for 15 min prior to the experiment. Conditioning training was conducted 8 times, once per day with alternative administration of morphine and saline. Rats injected with saline (10 mL/kg (i.p.)) and morphine (10 mg/kg (i.p.)) were placed into different compartments for 50 min. Tests of conditioning were conducted 1 day after the last conditioning session. For test sessions, the guillotine door separating the two compartments was opened and the rats were allowed to freely access to the entire apparatus for 15 min without any restriction. The time spent in morphine and saline paired environment was recorded and analyzed by RatTrack software. Extinction training was performed 8 times once per day after conditioning test. All the rats were administrated with saline only, then alternatively confined to each of compartments for 50 min. Tests of extinction were performed 1 day after the last extinction session. On the day after extinction test, the rats were treated with a priming injection of morphine (2.5 mg/kg), then allowed free access to the entire apparatus for 15 min, the time spent in morphine and saline paired environment was recorded and analyzed.

After recording in each phase, the rats (9 of each phase) were sacrificed immediately and their brains were dissected out and cut into slices as described by Paxinis and Watson.[16] The PFC was separated from each slice and frozen by liquid nitrogen. The crude postsynaptic density (PSD) fraction was isolated from PFC based on a modified method of Ka Wan Li et al.[17] Briefly, pooled PFC of three rats was homogenized in homogenization buffer (5 mM Hepes, pH 7.4; 320 mM sucrose) containing protease inhibitors (10 mg/mL Aprotinin, 10 mg/mL Leupeptin, and 1 mmol/L PMSF). Cell debris and nuclei were removed by 1000× g centrifugation. The supernatant was spun for 20 min at 12 000× g, and then the resulting P2 pellet was suspended in a buffer (12 mM Tris-HCl (pH 8.1), 320 mM sucrose) and mixed with an equal volume of 1% Triton X-100, 320 mM sucrose. After stirring for 15 min, the mixture was centrifuged for 30 min at 33 000× g. The precipitate was resuspended in 320 mM sucrose, 0.5% Triton X-100, 5 mM Tris/HCl, pH 8.1. After another 15 min of stirring, the crude PSD proteins were pelleted by a 2 h centrifugation at 201 800× g. The crude PSD pellet was solubilized in lysis buffer (7 M urea, 2 M thiourea, 2% CHAPS, 20 mM Tris, pH 7.5, 0.5% DTT, and 0.5% IPG buffer 3−10 NL) for 1 h and followed by centrifugation. All steps were carried out at 4 °C. Protein concentrations were determined using the Bradford assay.[18] Samples were stored at −70 °C prior to electrophoresis.

2.4. 2-DE and Silver Staining. Isoelectric focusing (IEF) was performed according to manufacturer's instructions (Amersham Biosciences) with IPGphor isoelectric focusing system (Amersham Biosciences). A rehydration solution containing 6 M urea, 2 M thiourea, 2% CHAPS, 0.5% IPG buffer 3−10 NL-(nonlinear), 18 nM DTT, and a trace of bromophenol blue were added into 300 μg of crude PSD proteins to reach a final volume of 450 μL of solution and applied to 24 cm IPG strip (pH 3−10, NL). Rehydration and IEF were carried out on the IPGphor platform automatically at 20 °C, and the total number of IEF is 71 000−73 000 Vh. After IEF, the gels were equilibrated for 15 min in an equilibration buffer (50 mM Tris-HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS, and a trace of bromophenol blue) containing 1% DTT and followed by equilibration in the same buffer with 5% iodoacetamide for another 15 min. The equilibrated gel strip was handled to 11% SDS-PAGE and sealed with 0.5% agarose. SDS-PAGE was performed at constant power of 2.5 W/gel for 30 min, subsequently changed to 15 W/gel. The separated proteins were fixed and stained by MS-compatible silver staining following the protocol described previously.[19] Triplicate gels were run for morphine and saline treatment groups in each phase, respectively. To reduce variations, three parallel experiments were performed.

2.5. Image Acquisition and Statistical Analysis. The silver-stained 2-D gels were digitized scanned at an optical resolution of 600 dpi with Power Look 1000 scanner (Umax, Willich, Germany). The images were analyzed with Phoretix 2D 6.01 analysis software (Nonlinear Dynamic, Durham, NC). Analysis includes four procedures: spot detection, spot matching, subtraction background, and normalization. During spot detec-

tion, 18 gels which come from three parallel experiments were built as a group. The spot detection was initially performed with software automatically. As many spots would be omitted or could not be circled properly by the automatic mode, the spot detection was further corrected by manual mode. In the second step of spot matching, all the spots detected in each gel were matched to the correspondence in reference gels. The volume of the spot represents the expression amount of a protein spot, which was defined as the total intensities of all the pixels that make up the spot. The individual spot volume was normalized as a percentage of the total volume of all the spots detected on the gel to eliminate variations produced by silver staining. Statistical analyses were performed using one-way analysis of variance (ANOVA). Volume difference of spots between the morphine-treated group ($n = 9$) and saline group ($n = 9$) of each phase was considered significant at $P < 0.05$.

2.6. In-Gel Digestion. All differentially expressed proteins were manually excised from the gel with a dermal slicer. Each excised gel piece was washed twice with water and destained in a 1:1 solution of 30 mM potassium ferricyanide and 100 mM sodium thiosulfate until brownish color disappeared, then it was rinsed three more times in distilled water to stop the reaction. Subsequently, the gel piece was equilibrated with 100 mM ammonium bicarbonate for 10 min, dehydrated with acetonitrile, and dried in a Savant Speed-Vac system (Thermo Savant, Holbrook, NY). The gel piece was rehydrated in 10−20 μL of proteomics grade trypsin solution (20 μg/mL in 40 mM ammonium bicarbonate in 9% acetonitrile) for 30 min on ice, followed by addition of 25 μL of 25 mM ammonium bicarbonate buffer to completely immerse the gel piece. After incubation overnight at 37 °C, peptides on the gel piece were extracted with 90% acetonitrile, 10% trifluoroacetic acid for 15 min and 50% acetonitrile, 5% trifluoroacetic for another 15 min at RT and dried in a vacuum centrifuge. The extracted peptides were then dissolved again in 10 μL of 50% acetonitrile solution with 0.1% trifluoroacetic acid. Although the MS signal was weak, the peptides were treated with ZipTips (Millipore, Billerica, MA) according to the manufacturer's instructions.

2.7. **Matrix-Assisted Laser Desorption/Ionization-Time-of-Flight Mass Spectrometry (MALDI-TOF−MS) Analysis.** The peptide mixture derived from each gel piece was applied to a stainless steel sample plate and allowed to air-dry. All of the dried samples were analyzed using Voyager-DE STR MALDI-TOF mass spectrometer (ABI Applied System, Framingham, MA). Mass/charge ratios were measured in the reflector/delayed extraction mode with an accelerating voltage of 20 kV, grid voltage of 64.5%, and delayed time of 100 ns. Monoisotopic peptide masses were assigned, and TOF spectra were collected over the mass range of 800−4000 Da. Des-Argl-bradykinin and ACTH were performed as external calibration simultaneously as the samples to be measured. Additionally, the auto-digested peaks of trypsin were served as internal standards for mass calibration.

2.8. **Protein Identification and Database Searching.** Peptide mass fingerprinting (PMF) was searched against the NCBI protein database with the MASCOT search engine (http://www.matrixscience. com) to identify proteins. The search parameters were defined as follows: rattus, trypsin digest (allowed up to 1 missed cleavage), cysteines modified by carbamidomethylation, methionine modified by oxidization, and maximal mass tolerance of 100 ppm. Proteins were considered as significant match ($P < 0.05$) when MASCOT scores were higher than 58, which was defined by the search engine automatically.

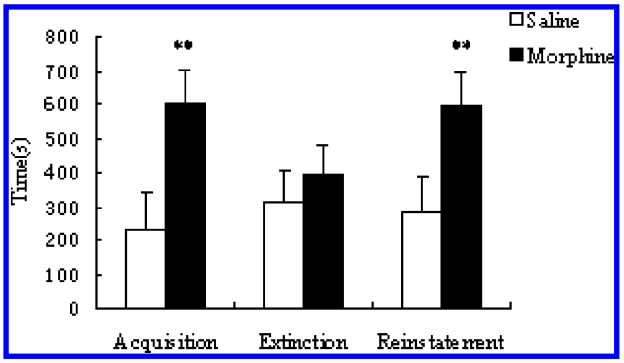

Figure 1. Statistics results of time spent in morphine-paired compartment in 15 min of conditioning ($n = 48$), extinction ($n = 33$), and reinstatement tests ($n = 24$) between morphine and saline treated groups based on three parallel behavioral experiments (mean ± SD). *$P < 0.05$ and **$P < 0.01$, compared with saline treatment.

2.9. Western Blot Analysis. The crude PSD proteins were suspended in 2-D lysis buffer in the absence of IPG buffer and bromphenol blue. After measuring the concentration with Bradford, 50 μg of these PSD proteins was mixed with 2× SDS sample buffer, boiled for 3 min, and then loaded to a 10% mini SDS-gel. After electrophoresis at 120 V for 1 h 20 min, the proteins in the gels were transferred to nitrocellulose membrane in transfer buffer containing 20% methanol at 100 V for 2 h. Membranes were blocked at room temperature for 2 h in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO4, 2 mM KH_2PO4) containing 5% nonfat milk to prevent nonspecific binding of reagents and then incubated in anti-CNPase (1:200 dilution) at room temperature for 2 h. Following washing in TBST (50 mmol/L Tris-Cl, pH 7.6, 150 mmol/L NaCl, 0.1% Tween20) for 20 min, the membrane was incubated with IRDyeTM800CW Conjugated secondary antibody (1:5000 dilution) for 1 h at room temperature. After washing in TBST for 40 min, the membranes were scanned with Odyssey (LI-COR Biosciences, Lincoln, NE) to detect and quantify the target protein. Anti-β-actin was also blotted with membrane as internal control.

3. Results

3.1. Proteins Differentially Expressed in Morphine Induced CPP. To identify the proteins differentially expressed in morphine addiction, the protein expression level and constitute in the synaptic fraction of PFC from morphine and saline pretreated rats were analyzed by 2-DE analysis. To obtain statistically reliable results, we carried out three parallel CPP experiments. Of the total 48 rats in morphine treated group (sixteen for each experiment), 42 could acquire the morphine-paired preference after training. Nine rats were sacrificed after conditioning test for 2-D analysis, and the remaining 33 would be taken into extinction training. Nine rats were sacrificed after extinction test for 2-D analysis and the remaining 24 rats were performed reinstatement by low dose of morphine priming. Eighteen rats could be successfully reinstated. At last, nine rats were sacrificed for 2-D analysis. Similarly, nine rats from each of CPP phases (acquisition, extinction, and reinstatement) in the saline-treated group were sacrificed for 2-D analysis. Taken together, a total of 54 rats from three parallel behavioral experiments were used for further proteomic study. Figure 1 shows the statistics result of three parallel CPP experiments.

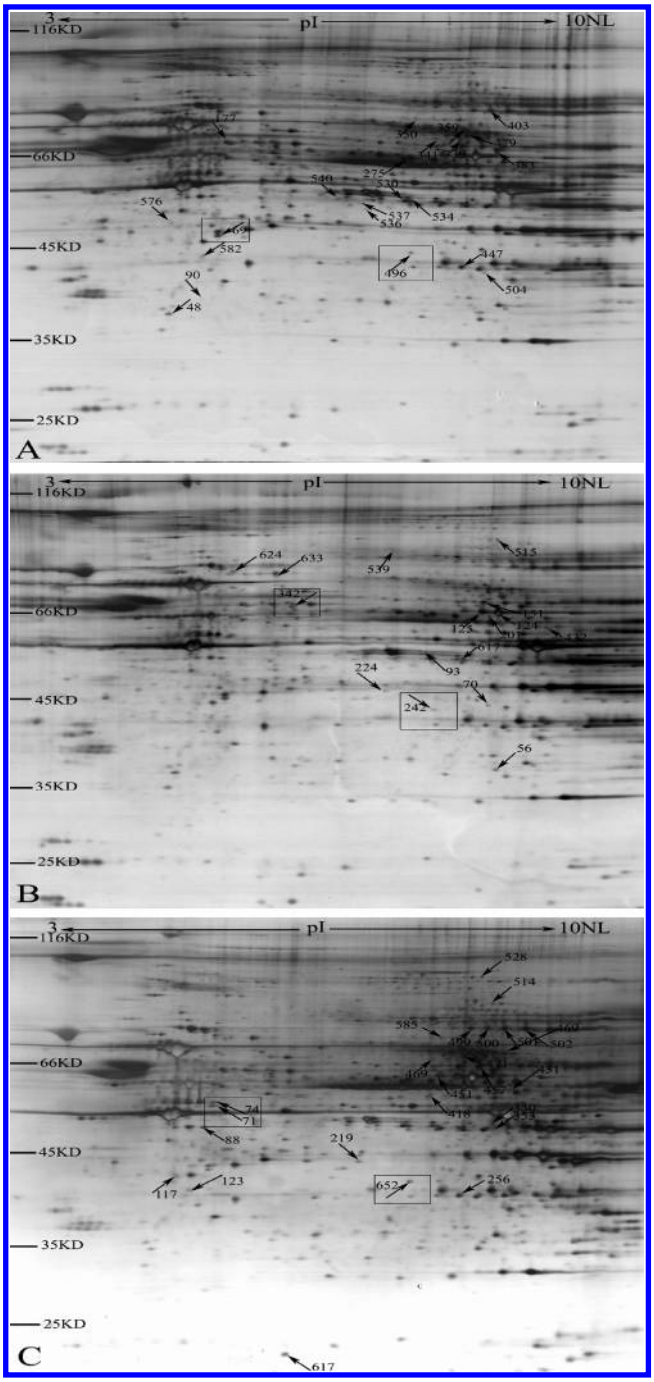

Figure 2. Representation of identified differential protein spots in 2-D gel templates from acquisition, extinction, and reinstatement phases in morphine treated groups. The protein samples were separated on a pH 3−10 nonlinear IPG strip, followed by 11% SDS-PAGE and silver staining. Upward or downward arrows indicate that the proteins were up-regulated or down-regulated by morphine treatment compared with saline treatment. The number next to the spot is the index number in the reference gel, and A, B, and C on the left bottom of each gel represent acquisition, extinction, and reinstatement phases of CPP in which the protein was differentially expressed. The spots in the rectangle area will be zoomed in Figure 3.

Pooled proteins of three rats from either morphine or saline treatment in each phase were analyzed. By silver staining of 2-D gel, approximately 800 spots per gel were observed (Figure 2). Proteins that were differentially expressed in 2-D gels were selected on the basis of the criteria mentioned in the Method Section 2.5. A representative of differentially expressed proteins was shown in Figure 3. Overall, there are 80 proteins that showed significant expression differences in three CPP phases between morphine and saline treatment after three times of experiment repeats. Among them, one showed significant expression difference in all three CPP phases, whereas the remaining 79 were different in only one or two CPP phases.

3.2. Identification of Proteins Differentially Expressed in Morphine Induced CPP. To identify 80 differentially expressed proteins, their corresponding spots were excised from the silver-stained gels, then digested with trypsin and identified by MALDI-TOF analysis and mascot searching database. As summarized in Table 1, a total of 58 unique differentially expressed proteins were successfully identified: 21 proteins in acquisition phase, 14 proteins in extinction phase and 23 proteins in reinstatement phase. The remaining 17 protein spots could not be identified by PMF because of low signals in the spectrum, and 5 protein spots were shown as mixtures from the MASCOT searching. This phenomenon of multiple proteins in one spot was also observed in other studies.[17,20]

By using bioinformatics analysis, the identified proteins were further classified into 6 functional groups: energy metabolism, signal transduction, synaptic transmission, cytoskeletal proteins, chaperones, and local synaptic protein synthetic machinery. Most of the identified proteins in this study were similar with those identified in the purified PSD.[17,20]

3.3. Confirmation of Altered Proteins by Western Blot Analysis. To verify the reliability of proteomics analysis, we carried out Western blot analysis of a representative protein, cyclic nucleotide phosphodiesterase 1 (CNPase1), which showed significant changes in expression in three phases of CPP. As shown in Figure 4, there were 2.1× elevation in the acquisition phase, 1.6× decrease in the extinction phase and 1.4× increase again in the reinstatement phase after at least three times repeats. Although moderate, the observed differences by Western blot were consistent with the results of 2-DE and silver-staining, in which the difference of CNPase1 was 2.4× elevation, 2.1× decline, and 1.5× increase during the respective three phases. The data indicates that the changes in protein expression identified by proteomic analysis of synaptic fraction of PFC in the three phases of CPP are reliable.

4. Discussion

2-DE based proteomics technology is an unbiased method which can simultaneously study the complex biological functions involving large numbers of genes at protein level. It overcomes the disadvantage of conventional biochemical methodologies which can only analyze one or a few proteins at one time.[21] Recently, the proteomics methodology as a powerful tool has been increasingly applied to the studies of morphine and other psychoactive drug dependence[22−29] such as proteomic analyses of synaptic plasma-membrane subproteome in morphine-dependent rats[23] and nucleus accumbens of rat with intermittent morphine treatment.[24] However, similar analysis in PFC region covering different stages of morphine addiction has not been reported yet.

In our current study, we employed the proteomic approach to identify the synaptic proteins in rat PFC associated with morphine induced addiction using the CPP model. A total of 80 proteins were identified, and 58 of them were further confirmed by MS analysis. According to their biological functions, the 58 identified proteins can be classified into 6

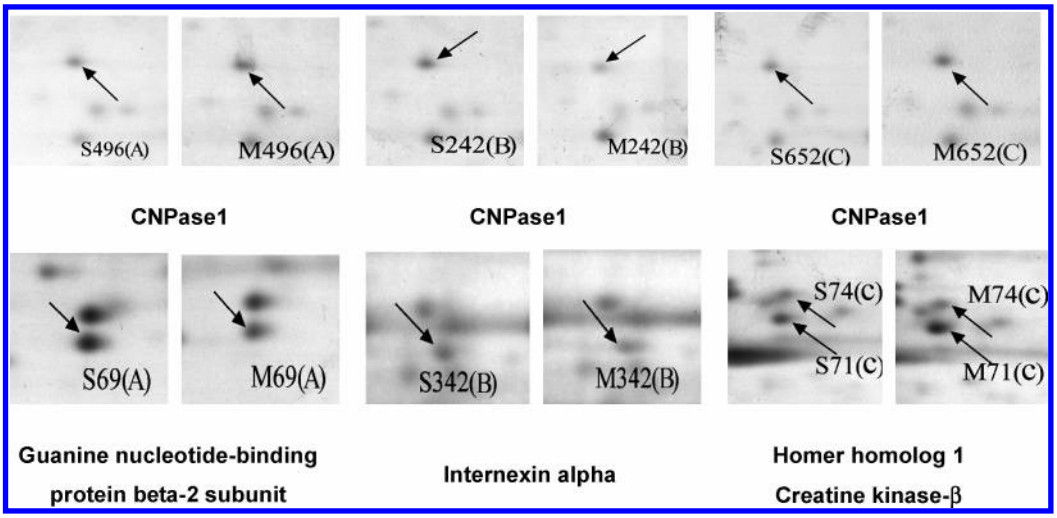

Figure 3. Zoomed image of differentially expressed proteins: CNPase1 (496, 242, 652), Guanine nucleotide-binding protein beta-2 subunit (69), Internexin alpha (342), Homer homolog 1 (71), Creatine kinase-β (74). A, B, and C in the bracket represent acquisition, extinction, and reinstatement phases of CPP in which the protein was differentially expressed. S and M represent saline and morphine treatment, respectively.

classes: energy metabolism, signal transduction, synaptic transmission, cytoskeletal proteins, chaperones, and local synaptic protein synthetic machinery. Although 8 morphine-responsive proteins, or closely related isoforms were reported in recent published proteomic analysis of morphine dependence, exemplified as ATPase H^+ transporting V1 subunit B isoform 2, guanine nucleotide binding protein beta polypeptide 2-like 1, guanine nucleotide-binding protein beta-2 subunit, cyclic nucleotide phosphodiesterase 1, glutamate dehydrogenase 1, calcium/calmodulin-dependent protein kinase II alpha, creatine kinase-B, Lamin-A, most of differential expressed proteins detected in this study were different from the prior analyses.[22−26] Thus, our study provides new insight to understand the synaptic molecular basis of morphine induced CPP.

A number of proteins involved in mitochondrial function and energy metabolism were found to be altered after morphine treatment. We observed that proteins involved in citric acid cycle and oxidative phosphorylation were down-regulated in the three phases of CPP procedure, such as ATPase, H^+ transporting, V1 subunit B, isoform 2 (Atp6v1b2), NADH dehydrogenase (ubiquinone) flavoprotein 2 (Ndufv2), MMRP19-like-protein (Mmrp19), acetyl CoA transferase-like-protein (ACAT), dihydrolipoamide S-acetyltransferase (E2 component of pyruvate dehydrogenase complex) and Aconitase 2, mitochondrial, which implied that energy synthesis was regulated during chronic morphine exposure. As a result for compensation of energy requirement, one of pyruvate kinase, similar to pyruvate kinase (EC 2.7.1.40) isozyme M2 (RGD1561681), was increased in the acquisition and reinstatement phases, respectively. Moreover, a protein, Acyl-Coenzyme A thioesterase 2, mitochondrial (LOC302640), which manifested the decomposition of fatty acids, was also elevated. Therefore, our findings are consistent with those derived from a cDNA microarray study showing that even a single injection of morphine transiently down-regulated a group of genes that encode mitochondrial oxidation proteins.[30] The expression changes of these proteins strongly suggest that mitochondrial oxidation dysfunction could be one of major molecular processes in morphine-induced dependence, withdraw and relapse. Furthermore, Prps1 protein and methylenetetrahydrofolate dehydrogenase ($NADP^+$ dependent), methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthase (Mthfd1) were decreased in the acquisition and reinstatement phases respectively in this study, which revealed other types of metabolism, such as nucleotide, amino acid, and folic acid were also influenced by morphine treatment. Therefore, it seems that morphine administration leads to disorder in the entire metabolic system.

It has been reported that 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase) is one of downstream signal molecules of cAMP-mediated pathway. An elevation of intracellular cAMP concentration significantly increased transcription of CNPase1 mRNA through binding to nucleotides −126 and −102 of CNPase1 promoter region.[31] Our results derived from both western blot and 2-DE analyses indicated a dynamic alteration of CNPase1 in the three phases of CPP model: significantly increased after morphine repeated treatment, followed with a decrease after morphine withdrawal and up-regulated again after morphine priming challenge. These results suggest morphine treatment could modulate CNPase by cAMP-mediated signal pathway. CNPase is nonspecifically associated with PSD fraction, a marker for the PSD,[32] and CNPase is a membrane bound microtubule associated protein that can link tubulin to membranes and may regulate cytoplasmic microtubule distribution.[33] Thus, we speculate that long-term morphine stimulus could change the structure of neural cell and synaptic linkage by CNPase-mediated cytoskeleton redistribution.

Glutamate receptor and voltage-dependent calcium channel are known to be involved in triggering major forms of long-term potentiation (LTP) and long-term depression (LTD),[34] which may play an important role in the development of drug-related behavior. It has been shown that transient Ca^{2+} entry through NMDA channel that occurs during LTP induction stimulates the persistent autophosphorylation and activation of CaMKII, whereas dephosphorylation of CaMKII participates in AMPA receptor mediated LTD.[35] We found that calcium/calmodulin-dependent protein kinase II alpha (CMKIIα), and its isoform1 were increased in the extinction phase and reinstatement phase respectively, suggesting CMKIIα could be one of molecular and celluar bases of morphine induced synaptic plasticity. Another identified protein Homer homolog 1, a component of the metabotropic glutamate signaling

Table 1. Differentially Expressed Proteins Identified by MALDI-TOF−MS Analysis

index no.	accession no.	mass	p*I*	cov. (%)	peptide match	MOWSE score	protein description	expression change[a] acquisition	extinction	reinstatement
							Energy metabolism			
177	gi|55716047	56857	5.57	51	59 (23)	210	ATPase, H+ transporting, V1 subunit B, isoform 2	0.509		
339 (471)	gi|62662888	58264	7.15	22	19 (10)	112	PREDICTED: similar to pyruvate kinase (EC 2.7.1.40) isozyme M2 - rat	1.833		1.668
48	gi|51092268	27703	6.23	47	12 (10)	182	NADH dehydrogenase (ubiquinone) flavoprotein 2	0.615		
90	gi|27702700	27605	6.45	37	7 (7)	137	PREDICTED: similar to MMRP19	0.421		
576	gi|54035342	41538	6.86	26	27 (7)	73	Similar to acetyl CoA transferase-like	0.597		
633	gi|78365255	67637	8.76	21	42 (12)	83	Dihydrolipoamide S-acetyltransferase (E2 component of pyruvate dehydrogenase complex)		0.589	
432	gi|55250049	50753	8.8	20	18 (10)	111	Acyl-Coenzyme A thioesterase 2, mitochondrial		1.545	
56	gi|62650342	42643	9.91	20	17 (8)	100	PREDICTED: similar to LRRGT00014		0.693	
70	gi|50927633	35325	6.51	30	21 (7)	76	Prps1 protein		0.686	
117	gi|74220526	56857	5.57	46	48 (19)	181	Unnamed protein product			1.614
514	gi|38541404	86121	7.87	31	42 (20)	183	Aconitase 2, mitochondrial			0.734
528	gi|11968082	101616	7.05	24	30 (20)	213	Methylenetetrahydrofolate dehydrogenase (NADP+ dependent), methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate			0.49
350	gi|1334163	39590	6.78	50	29 (13)	160	Unnamed protein product			0.736
353	gi|1334163	39590	6.78	59	37 (14)	161	Unnamed protein product			0.787
							Signal transduction			
479	gi|62663767	42828	6.99	25	32 (20)	120	PREDICTED: similar to WD repeat domain 37	1.864		
504	gi|15559817	35511	7.6	54	20 (11)	158	Guanine nucleotide binding protein(G protein), betapolypeptide 2-like 1	2.981		
69	gi|20357529	38048	5.6	57	38 (14)	163	Guanine nucleotide-binding protein, beta-2 subunit	0.571		
496 (242, 652)	gi|57977323	47638	9.03	46	22 (13)	175	Cyclic nucleotide phosphodiesterase 1	2.243	0.477	1.541
582 (123)	gi|55742832	36213	5.42	40	15 (12)	181	Annexin A4	0.264		0.505
275	gi|76623452	54651	6.61	32	24 (14)	155	PREDICTED: similar to Calcium/calmodulin-dependent protein kinase type II alpha chain (CaM-kinase II alpha chain) (CaM kinase II alpha subunit) (CaMK−II alpha)	2.003		
124	gi|6980956	61731	8.05	52	49 (27)	296	Glutamate dehydrogenase 1		1.591	
125	gi|6980956	61731	8.05	51	61 (27)	234 (134)	Glutamate dehydrogenase 1		1.673	
201	gi|21619328	54651	6.61	39	14 (12)	188	Calcium/calmodulin-dependent protein kinase II alpha		1.61	
617	gi|6753250	22740	6.45	45	11 (7)	116	Calcium/calmodulin-dependent protein kinase II alpha isoform 1			1.434
71	gi|203476	40883	5.32	47	30 (14)	173	Creatine kinase-B			1.311
74	gi|13928988	41394	5.39	59	57 (22)	230	Homer homolog 1			1.664
451	gi|56200	61731	8.05	36	31 (15)	139	Unnamed protein product			1.376
							Synaptic transmission			
350	gi|57033186	67925	6.49	32	31 (17)	166	Syntaxin binding protein 1	1.626		
383	gi|112350	52822	7.62	37	22 (10)	121	Synapsin IIb	2.526		
403 (501)	gi|77404242	63702	8.73	42	52 (17)	130	Synapsin 2 isoform 1	2.153		1.288
431	gi|12621100	45132	7.19	24	11 (8)	123	Double C2, alpha			0.666
457	gi|77404242	63702	8.73	37	29 (14)	158	Synapsin 2 isoform 1			1.35

Table 1 (Continued)

index no.	accession no.	mass	p*I*	cov. (%)	peptide match	MOWSE score	protein description	expression change[a] acquisition	reinstatement	extinction
499	gi|112350	63702	8.73	30	19 (11)	133	Synapsin 2 isoform 1			1.663
500	gi|77404242	63702	8.73	30	14 (12)	181	Synapsin 2 isoform 1			1.39
502	gi|77404242	63702	8.73	48	44 (18)	177	Synapsin 2 isoform 1			1.624
585	gi|73760415	67925	6.49	40	21 (20)	285	Syntaxin binding protein 1 isoform b			0.494
							Cytoskeletal proteins			
447 (256)	gi|62655196	39974	6.61	31	17 (13)	118	PREDICTED: similar to actin related protein 2/3 complex subunit 2	0.577		0.757
530	gi|56789539	44990	6.3	27	20 (9)	98	ARP2 actin-related protein 2 homolog (yeast) (predicted)	0.728		
341 (469)	gi|62659058	71567	6.65	24	25 (13)	108	PREDICTED: similar to Coronin, actin binding protein 1C	3.316		1.823
93	gi|51858705	57619	5.89	14	12 (6)	68	Archain		1.626	
342	gi|9506811	56253	5.2	26	24 (12)	127	Internexin, alpha		0.736	
539	gi|1346413	74564	6.54	47	78 (32)	205	Lamin-A		2.341	
88	gi|13928838	39468	5.34	52	23 (15)	197	Tropomodulin 2			1.38
							Local synaptic protein synthetic machinery			
534	gi|13435897	37987	6.66	37	15 (7)	94	Poly(rC) binding protein 1	1.913		
540	gi|34878862	34976	6.07	23	20 (6)	72	PREDICTED: similar to Transcriptional activator protein PUR-alpha (Purine-rich single-stranded DNA-binding protein alpha)	0.691		
536	gi|62660090	2E+05	6.43	30	25 (12)	96	PREDICTED: similar to zinc finger motif enhancer binding protein 2	2.357		
537	gi|62651671	30431	6.83	34	30 (14)	90	PREDICTED: similar to Sfrs7 protein	1.519		
515	gi|61097941	83414	6.81	29	29 (17)	165	DEAD (Asp-Glu-Ala-Asp) box polypeptide 1			1.384
							Chaperones			
224 (219)	gi|62665798	49248	5.24	19	30 (9)	80	PREDICTED: similar to heat shock transcription factor 2 binding protein		0.691	0.677
624	gi|92355	69785	5.44	32	31 (11)	103	DnaK-type molecular chaperone hst70		0.652	

[a] Expression change is the volume of spot of morphine treatment divided by that of saline treatment.

pathway, was up-regulated in reinstatement phase. Homer belongs to scaffold protein, one of major kind of components in PSD. Previous study has reported that Homer1b/c can link with metabotropic glutamate receptor 5(mGluR5) to activate extracellular signal-regulated protein kinases 1 and 2 (ERK1/2). This signal pathway has the ability to phosphorylate two transcription factors, Elk-1, and cAMP response element-binding protein (CREB), and thereby facilitates c-Fos expression to mediate the transcriptional regulation.[36] Furthermore, Homer1 and Homer2 proteins have been reported to be regulated by acute and chronic cocaine administration. A deletion of Homer1 or Homer2 in mice causes increased sensitivity to cocaine-induced locomotion, conditioned reward, and augmented extracellular glutamate in nucleus accumbens which are the same as withdraw from repeated cocaine administration.[37] Thus, we speculate that homer could participate in morphine addiction by regulating the metabotropic glutamate signal transduction.

Synapsins are associated with cytoplasmic surface of small synaptic vesicles, binding to cytoskeleton and contribute to regulation of neurotransmitter release.[38−40] They are neuron-specific phosphorproteins which serve as major brain substrates for cAMP-dependent and Ca^{2+}/calmodulin-dependent protein kinases.[41,42] In this study, synapsin 2 isoform 1 was markedly up-regulated in the acquisition and reinstatement phases and synapsin IIb was also up-regulated by morphine priming after withdraw. Moreover, many synapsin spots were found in a line in the 2-D gels which supposedly represent different post-modification forms of the proteins. The observation in our study was consistent with the one derived from previous study.[17] Our results manifest morphine induced cAMP-dependent and Ca^{2+}/calmodulin-dependent protein kinases alteration would modulate the expression and phosphorylation of synapsins.

Consistent with previous results,[43] we observed that cytoskeletal proteins can be regulated by morphine administration. As an example, alpha-internexin was decreased in the extinction phase. Alpha-internexin plays an important role in neurite outgrowth and regulates expression of other neurofilaments during neural development.[44] The down-regulation of alpha-

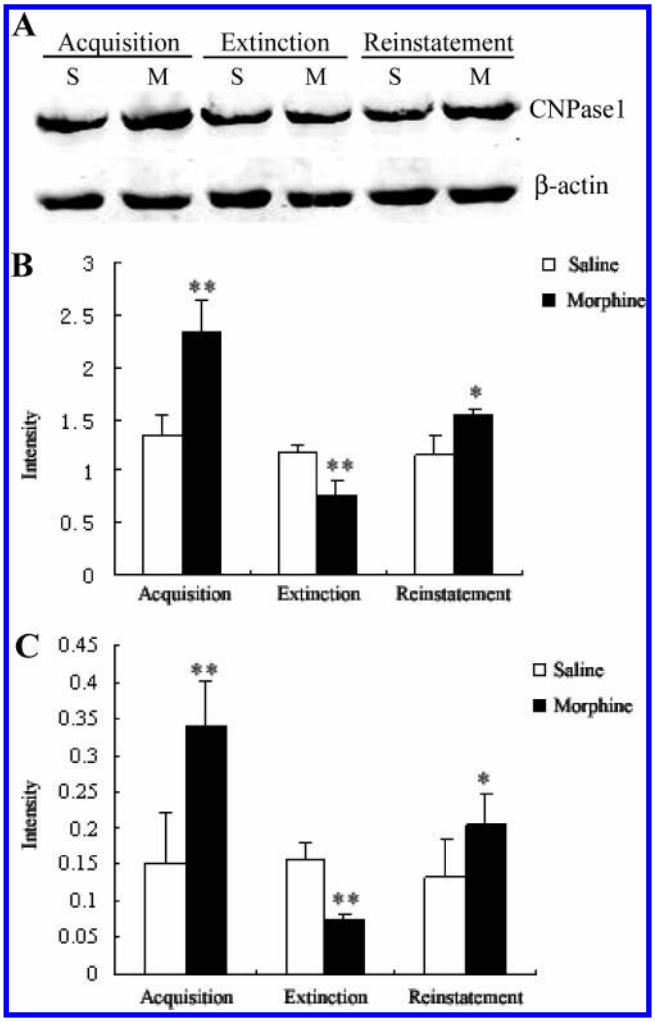

Figure 4. Effect of morphine treatment upon CNPase1 expression in crude postsynaptic density (PSD) of prefrontal cortex (PFC) in each phase of conditioned place preference (CPP). Western blot analyses were performed using anti-CNPase1 and anti-β-actin antibodies as described in Section 3.3. An image of Western blot analysis scanned with Odyssey is shown in (A) (S, saline; M, morphine). CNPase1 level was quantified using Odyssey software and was normalized by β-actin level (B). Spot intensity was derived from silver staining 2-D gels (C). Data was indicated as means $\pm$ SD from three independent experiments. $^*P < 0.05$ and $^{**}P < 0.01$, compared with saline treatment.

internexin observed in current study suggests that morphine administration may induce a disorganization of neuronal regeneration and morphogenesis through regulation of cytoskeleton and its interaction proteins. This study also showed that tropomodulin2 increased in the reinstatement phase. Tropomodulin2 is important for maintenance of cytoskeleton by binding with neuron-specific isoform of tropomyosin to block elongation and depolymerization of actin filaments. Tropomodulin2 functions to not only maintain the cytoskeleton but also participate in nerve−nerve synaptic transmission and positive regulation of G-protein coupled receptor protein signaling pathway. Taken together, the altered regulation observed in cytoskeletal proteins suggests modification in synaptic transmission and neural morphology, thus underlie neuroadaptations to chronic morphine administration. In addition, several proteins identified in this study have unknown biological functions or are not reported to associate with drug dependence. They possibly represent novel candidates and their roles in morphine addiction are worth to be further characterized.

5. Concluding Remarks

In this study, proteomics is applied to investigate the effect of morphine dependence, withdraw, and relapse to the expression of synaptic proteins in PFC. A total of 80 proteins showed significant difference after morphine treatment compared to saline by using 2-DE, however, 58 proteins were identified by mass spectrum and database search. Some of the identified proteins are associated with important biological functions, such as energy metabolism, signal transduction, synaptic transmission, and cytoskeleton formation involved in morphine induced neuroplasticity. Therefore, this work provides a frame work for further investigation of the molecular adaptation mechanisms underling chronic morphine dependence, withdraw, and relapse which further contribute to the associated long-lasting behavioral sensitization.

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