Comparative Proteomic Analysis of the Nucleus Accumbens during Extinction and Reinstatement of Morphine Dependence

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ABSTRACT

Background: *The aim of this study was to detect differentially expressed proteins in the nucleus accumbens between the states of extinction and reinstatement of morphine addiction. Numerous studies on the neurobiological mechanisms concerning drug craving and relapse have been reported to date, but data on their relationship with the underlying key molecular mechanisms involved remain limited.*
Methods: *In this study, 40 male Sprague-Dawley rats were equally randomized into a saline group and a morphine group. Both groups received drug self-administration training, after which extinction models were established naturally. The groups were further divided into two subgroups for extinction and reinstatement tests. Cerebral nucleus accumbens masses were measured for total protein extraction. Two-dimensional electrophoresis was performed to determine differential protein spots. These differential proteins were then enzymolysed and identified using mass spectrography.*
Results: *The proteins were classified as fatty acid-binding protein, serine/threonine protein phosphatase 2A catalytic subunit beta isoform, serine/threonine protein phosphatase 2A catalytic subunit alpha isoform, serine/threonine protein phosphatase 2A regulatory subunit B² subunit gamma or heat shock protein 90 co-chaperone CDC37.*
Conclusion: *Significant changes in five proteins were detected between extinction and reinstatement. These proteins are correlated with phosphorylation and the tricarboxylic acid cycle.*

Keywords: Extinction, morphine craving, nucleus accumbens, proteomics, reinstatement

Análisis Proteómico Comparativo del Núcleo Accumbens durante la Extinción y Recaída de la Dependencia a la Morfina

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RESUMEN

Antecedentes: *El objetivo de este estudio fue detectar las proteínas diferencialmente expresadas en el núcleo accumbens entre los estados de extinción y recaída de la adicción a la morfina. Hasta la fecha se han reportado numerosos estudios en relación con los mecanismos neurobiológicos del deseo incontenible y recaída en el consumo de drogas, pero los datos sobre su relación con los mecanismos moleculares fundamentales subyacentes implicados, siguen siendo limitados.*
Método: *En este estudio, 40 ratas machos Sprague-Dawley fueron por igual asignadas de manera aleatoria a un grupo salino y un grupo de morfina. Ambos grupos recibieron entrenamiento de autoadministración de drogas, después de lo cual se establecieron modelos de extinción de manera natural. A su vez, los grupos fueron luego subdivididos en dos subgrupos para realizar pruebas de extinción y recaída. Se procedió a medir las masas cerebrales del núcleo accumbens para la extracción total de proteína. Se realizó una electroforesis bidimensional para determinar manchas proteicas diferenciales. Estas proteínas diferenciales fueron entonces sometidas a enzimólisis e identificadas mediante espectrografía de masa.*

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Resultados: *Las proteínas fueron clasificadas como proteína de unión a ácidos grasos, isoforma beta de la subunidad catalítica serina-treonina proteína fosfatasa 2A, isoforma alfa de la subunidad catalítica serina-treonina proteína fosfatasa 2A, subunidad gamma subunidad B" de la serina-treonina proteína fosfatasa 2A, o la proteína CDC37 cochaperona 90 de choque térmico.*
Conclusión: *Se detectaron cambios significativos en cinco proteínas entre la extinción y la recaída. Estas proteínas están correlacionadas con la fosforilación y el ciclo del ácido tricarboxílico.*

Palabras claves: Extinción, deseo incontenible por la morfina, núcleo accumbens, proteómica, recaída

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INTRODUCTION

Drug dependence and abuse have become a public health hazard worldwide. Although controlling drug addiction and blocking abused drug craving are known to be important steps in abuse elimination, a high relapse rate in prevention and cure has long been puzzling scholars. Thorough clarification of the mechanism underlying drug dependence relapse is crucial to solving this problem.

According to current theories, the dynamic balance between the reward and aversion processes of the central reinforcement system plays a critical role in drug dependence relapse. By contrast, the synclastic process theory of Wise and Bozarth posits that drug administration activates the mesencephalic dopamine system, which is responsible for drug-seeking, regardless of whether or not withdrawal symptoms appear after drug withdrawal.

Numerous studies on the neurobiological mechanisms concerning drug craving and relapse have been reported in the literature. These studies primarily focussed on neurotransmitters, nervous pathways and the central location associated with the reward circuit of the mesolimbic dopamine system [which is composed of such regions as the ventral tegmental area, *nucleus accumbens* (NAc) and amygdala] (1, 2), involving endogenous opioid peptides (3, 4), the noradrenergic system (5, 6), amino acids neurotransmitters (7) and corticotropin-releasing factors (8 – 10), among others. However, the underlying key molecular mechanisms that correspond to craving extinction and reinstatement remain poorly understood. Further research is needed to determine not only the similarities and differences between these mechanisms but also the targets of craving elimination, drug-seeking potential elimination and reinstatement control.

In this study, drug self-administration animal models were successfully established at an early stage. These models were then reinforced for the establishment of connections between their behaviours and drugs by combined drug injections. At the stage of drug withdrawal, drug injections were no longer given to quench the operant responses. At the stage of relapse, the operant models were reinstated through drug ignition stimulation. In addition, light stimulation was introduced throughout the experiment. The combination of light stimulation and drug ignition successfully helped simulate the initial behavioural characteristics of human relapse.

Deep exploration into the key proteomic differences between the states of extinction and reinstatement is essential for early diagnosis and treatment of morphine dependence. In this study, the proteomic method was adopted to explore the molecular mechanisms underlying different states of morphine dependence. The expression profiles of NAc proteins were compared among the physiological saline, morphine extinction and morphine reinstatement groups, and the electropherograms of these groups were analysed using the PDQuest 8.0 software programme.

MATERIALS AND METHODS

Animals

Forty male Sprague-Dawley rats weighing between 230 and 260 g and of clean grade were used in this study. They were maintained under a 12-hour light/12-hour dark cycle (lights on at 8 am) and in drafty animal houses from one week before the experiment. The environmental temperature was maintained between 22 and 24 °C, and the animals were allowed *ad libitum* access to food and water. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee of the First Affiliated Hospital of Chongqing Medical University.

Animal model establishment

The rats were subjected to venous cannulation in the supine position after pentobarbital sodium administration (40 mg/kg). In total, 0.2 ml of benzylpenicillin for anti-infection and 0.1 ml of heparin sodium solution for anticoagulation were given *via* the catheter for three consecutive days. After seven days of recovery, the animals were caged individually. At one week after catheter insertion, vests were put on the rats. All animals were equally randomized into physiological saline and morphine self-administration groups according to weight. The training lasted 14 days, for four hours per day, in a dark room.

Training programmes

Rats from the two groups were separately placed in rat self-administration activity detection boxes. The FR1 programme (*ie* injection of physiological saline or morphine was given after each nose poke) was performed for 14 consecutive days. Data on valid nose poke count, invalid nose poke count, morphine injection count and activity were recorded automatically by a computer.

Rats from both groups were placed in a dark training cage. They were allowed free access to water, but not food, during the training. The animals received two hours of extinction training per day for 13 consecutive days. Data on valid nose poke count, invalid nose poke count and activity were recorded automatically by a computer.

Both groups were further divided into a relapse group and an extinction group. The extinction groups received one hour of extinction training, whereas the relapse groups received training according to the CSH-one hour programme. Data on valid nose poke count, invalid nose poke count and activity were recorded.

Nucleus accumbens protein extraction

The rats were anaesthetised intraperitoneally with 60 mg/kg 3% pentobarbital sodium and immediately decapitated. Their brains were placed in pre-chilled physiological saline at 4 °C. Blood stains on the surface were washed off, and residual physiological saline was blotted. *Nucleus accumbens* masses were taken according to the profile of rat brain tissues as described by Paxinox and Watson (11) and Bao and Shu (12). The samples were immediately weighed, quickly frozen in liquid nitrogen for five minutes and then stored at -80 °C.

Two-dimensional electrophoresis (2-DE) and mass spectrography

Protein extraction: The sample in cryopreservation was taken out, washed with pre-chilled deionized water and swirled to remove blood cells. It was then frozen and thawed thrice for one minute each time. Next, the sample was thawed naturally at room temperature. Cocktail + DNA enzyme + RNA enzyme + PMSF were applied to obtain glass homogenate, which was then placed at 4 °C for 25 minutes. Lysate was added at a sample/lysate ratio of 1:12 (w/v). Next, the homogenate was placed at 4 °C for another 25-minute reaction. The obtained lysed sample solution was transferred into a silicified EP tube. After oscillation, the sample solution was centrifuged at 15 000 *g* for 30 minutes at 4 °C. The supernatant was collected, subpackaged and then stored at -80 °C.

Two-dimensional electrophoresis: 2-DE was per-formed according to the procedure described by Bio-Rad (USA). The in-gel sample application method was adopted. In total, 400 μg of the protein sample was diluted with 350 μL of hydrated sample buffer [containing 7 mol/L urea, 2 mol/L thiourea, 4% CHAPS, 65 mmol/L DTr, 2% (w/v) Bio-Lyte and 0.001% bromophenol blue]. The diluted sample was applied from left to right along the border of the median groove of the focussing plate. An IPG non-linear gel strip (pH 3-10, 17 cm) was placed over the sample solution in the focussing plate with the gel facing downward, which was further coated with 2–3 ml of mineral oil. Both 13-hour passive rehydration and isoelectric focussing were performed automatically at 17 °C, with a total voltage time integral of 70 000 Vh. After focussing, the strip was equilibrated in buffers I and II for 13 minutes each. The equilibrated strip was immerged in 1 × electrophoresis buffer for five seconds and then transferred on to the upper part of 12% SDS-PAGE gel. Close contact between the strip and SDS was ensured. The gel was embedded with 0.5% low-melting point agarose. Electrophoresis was done on a Protean II XI cell electrophoretic apparatus.

Staining and image analysis: Silver staining was conducted using a modified Sambronkd method. The stained 2-DE gels were scanned by the Amersham ImageMaster VDS-CL gel imaging system and analysed by PDQuest 7.4.0. Protein spots were detected, and changes in their expression were evaluated according to the changes in the normalized volume values.

Mass spectrography: Differential protein spots were cut and placed into EP tubes. They were decolourized, dried and enzymolysed for in-gel protein digestion. Matrixes and mass spectrographic target samples were applied. The pre-pared samples were analysed for peptide mass fingerprints (PMFs) using matrix-assisted laser desorption time-of-flight mass spectrometry. The obtained data were then searched against the database at http://www.prospector.ucsf.edu.

RESULTS

After repeated adjustments of the experimental scheme, the electropherograms of the same sample at different time points under the same conditions showed high repeatability. PDQuest 8.0 analysis revealed a matching degree of 85.23% among multiple electropherograms of the same sample and good separation of the 2-D electropherograms of all NAc masses. According to the analysis, the average protein spot counts in the saline extinction, saline reinstatement, morphine extinction and morphine reinstatement groups were 868 ± 27, 833 ± 24, 859 ± 38 and 838 ± 33, respectively. Twelve differential protein spots were found, and 10 were ultimately identified.

Two-dimensional electrophoresis

Comparisons of the 2-D electropherograms between the saline extinction and reinstatement groups showed that one spot disappeared in the reinstatement group compared with the extinction group (Figs. 1 and 2).

The 2-D electropherograms of the morphine extinction and reinstatement groups were compared, and the results showed that the expression of nine spots increased, the expression of one spot decreased and one new spot emerged

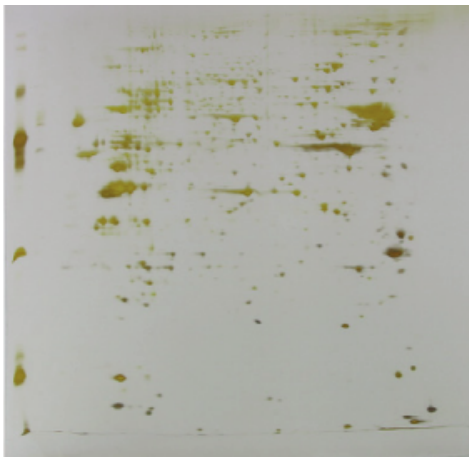

Fig. 1: Two-dimensional map of the saline extinction group.

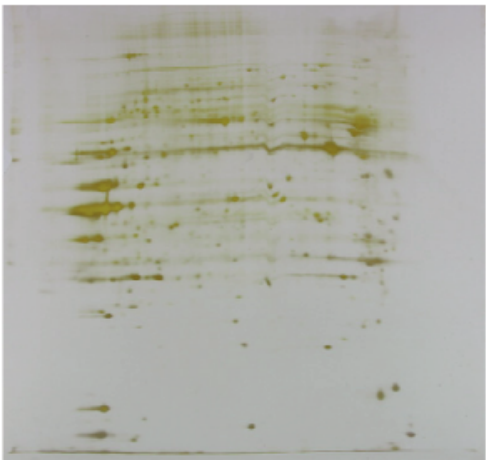

Fig. 2: Two-dimensional map of the saline reinstatement group.

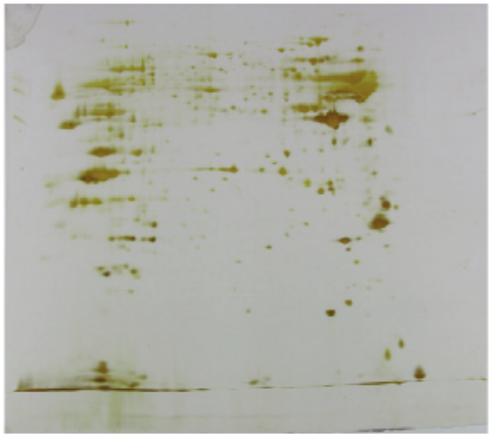

Fig. 3: Two-dimensional map of the morphine extinction group.

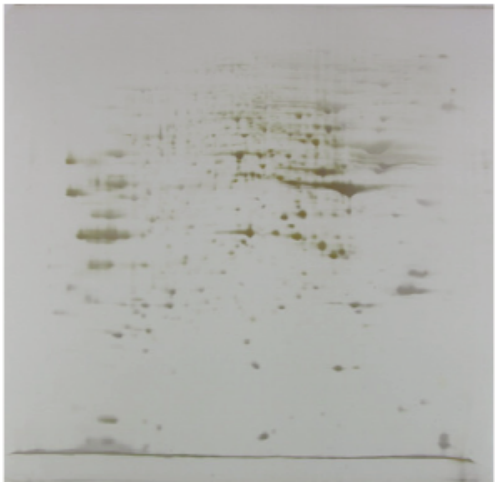

Fig. 4: Two-dimensional map of the morphine reinstatement group.

in the reinstatement group compared with the extinction group (Figs. 3 and 4). PDQuest 8.0 analysis revealed that spot 1 was expressed in both the extinction and reinstatement groups (although its expression was slightly different between the two groups, no significant difference was observed); spot 4 was a newly emerging differential protein spot in the reinstatement group; the expressions of spots 2, 3, 5, 6, 7, 8, 9, 11 and 12 in the reinstatement group increased compared with those in the extinction group, and the expression of spot 10 decreased.

PMF and databank-based identification

The results on the differential proteins by PMF and databank-based identification are summarized in the Table. The

Table: The result of protein identification

Spot number	% Cov	MW (Da)/pI	Accession	Search	Protein Name
2	43%	36123/5.21	P62716	Mascot/MS-Fit	PP2AB
3	40%	36156/5.30	P63331	Mascot/MS-Fit	PP2AA
4	32%	53379/6.0	Q6AXZ3	Mascot/MS-Fit	P2R3C
6	30%	44995/5.24	28151	Mascot/MS-Fit	CDC37
7	38%	40044/6.47	Q99NA5	Mascot/MS-Fit	IDH3A
8	24%	49984/5.02	Q3T153	Mascot/MS-Fit	SCHI1
9	22%	48226/5.79	Q99PW5	Mascot/MS-Fit	NEUR3
10	27%	21941/5.34	P35704	Mascot/MS-Fit	PRDX2
11	23.1%	25914/7.0	PO4905	Mascot/MS-Fit	GSTM1
12	27.1%	25703/6.9	PO8010	Mascot/MS-Fit	GSTM2

significant proteins included serine/threonine protein phosphatase 2A catalytic subunit beta isoform (PP2AB), serine/threonine protein phosphatase 2A catalytic subunit alpha isoform (PP2AA), serine/threonine protein phosphatase 2A regulatory subunit B^2 subunit gamma, heat shock protein 90 (HSP90) co-chaperone CDC37 and isocitrate dehydrogenase (IDH) subunit alpha mitochondrial precursor.

The PMFs of the differential proteins were obtained using mass spectrography. The SwissProt database was used, and the PMFs were retrieved using Mascot and MS-Fit software. MS-Fit is capable of limiting molecular weights and PI values, which allows for easier acquisition of outcomes. On the other hand, the outcomes of Mascot retrieval showed a high coverage rate between the amino acid sequences of the target proteins and those of some already known proteins in the database. This finding indicates a high possibility in protein matching, which has statistical significance.

DISCUSSION

This study found that changes in multiple NAc proteins occurred from the state of morphine dependence extinction to that of reinstatement. Further analysis revealed that these protein spots were PP2AB, PP2AA, CDC37 and IDH subunit alpha mitochondrial precursor, which are proteins correlated with physiological processes, such as energy metabolism, protein phosphorylation and stress, among others. The differential proteins identified in this study not only can provide a molecular biological basis for further explorations into the cause and pathogenesis of morphine dependence but also may serve as markers for the molecular diagnosis of such dependence.

In this study, IDH3A expression in the morphine reinstatement group was significantly higher than that in the morphine extinction group, but such significant difference was not observed between the saline groups. Isocitrate dehydrogenase is the rate-limiting enzyme in the tricarboxylic acid cycle. It catalyses isocitric acid to produce α-ketoglutaric acid and reduces nicotinamide adenine dinucleotide (NAD) or nicotinamide adenine dinucleotide phosphate (NADP) into NADH or NADPH. Isocitrate dehydrogenase plays an important role in energy metabolism and the synthesis of amino acids and vitamins as well as exerts a key regulatory effect on carbon flux distribution in the tricarboxylic acid cycle and glyoxylate bypass (13, 14). The results of this study suggest that the priming of morphine craving reinstatement significantly induces the production of IDH, which in turn increases the metabolic frequency of the tricarboxylic acid cycle. This suggestion is substantiated by the increased valid nose poke counts, activity levels and drug-seeking behaviours as well as the enhanced brain glycometabolism in rats in the state of morphine craving reinstatement. This conclusion is consistent with data reported in the literature, according to which opioid *mu* receptors activate the ATPase-dependent k^+ pathway *in vivo* to reinforce energy metabolism (15, 16). Moreover, the present study hypothesises that morphine increases ATPase and activates the k^+ pathway by increasing the production of the rate-limiting enzyme of the tricarboxylic acid cycle. Isocitrate dehydrogenase and gamma-aminobutyric acid catabolic enzyme increased in the brain stem and *pars thalamica* of rats seven days after treatment with morphine, and these enzymes were further activated at 14 and 21 days (17). These results indicate that long-term morphine administration potentially leads to functional changes in the tricarboxylic acid cycle and suggest that these changes may participate in the formation of morphine dependence in rats. Although the present study focussed on a different state of morphine dependence, it suggests that abnormal expression of energy metabolism-associated enzymes or coenzymes in brain tissues influences the normal level of glycometabolism and that glycometabolism dysfunction participates in the full range of the formation of morphine dependence, extinction and reinstatement. However, the mechanisms underlying these roles remain unclear.

This study also found that PP2AB and PP2AA resembled a railway distributed on the 2DE-PAGE and that they were two phosphorylation-modified proteins. Protein phosphorylation as a key signal process participates in neural information storage and affects protein phospholipases by equilibrating protein kinases (18). Serine/threonine protein phosphatase 2A is composed of a protein kinase and a protein phosphatidase coupled with the protein kinase; it catalyses reversible phosphorylation and dephosphorylation. Its flux is highly expressed in the neural system, and it is an important serine/threonine protein phosphatase in the brain. An increase in PP2A activity turns the balanced state between phosphorylation and dephosphorylation into a phosphorylation-dominating state, such as the phosphorylation of Ca^{2+}/calmodulin-dependent protein kinase type IV, which consequently leads to the activation of the apoptotic cascade reaction of neonatal brain tissues. By contrast, inhibiting the enzymatic activity of PP2A can inhibit apoptosis; therefore, the phosphorylation equilibrium in cells is crucial for normal physiological metabolism of cells and even determines their life and death (19).

This study attributes the regulation of the activity of PP2A to the following:

* Research has shown that glutamate receptors are noticeably upregulated in rats with morphine dependence and that the activation of glutamate receptors increases tumour necrosis factor (TNF) expression to regulate ceramide expression, which further upregulates PP2A activity (20). In the present study, the reinstatement of morphine craving activated glutamate receptors to upregulate PP2A activity and to increase its expression. In addition, the results suggest that an increase in PP2A and its apoptosis may lead to irreversible injury.
* PP2A performs an important role in the dephosphorylation of numerous phosphorylated proteins. The activation of protein autophosphorylation decreases PP2A

activity (21), and this process is crucial for maintaining the long-term potentiation of synaptic protein phosphorylation. When PP2A expression is upregulated, the phosphorylation level will be increased; this condition is correlated with fear and long-term memories (22). These findings suggest that an increase in PP2A activity may induce the reinforcement of heart addiction closely associated with long-term memory. Therefore, PP2A plays an important role in the relapse of morphine craving.

Moreover, this study found that CDC37 expression in the morphine reinstatement group was significantly higher than those in the morphine extinction group ($p < 0.01$) and saline reinstatement group. These results suggest that mental stress causes an increase in CDC37 expression. CDC37 is an auxiliary molecular chaperone specifically binding with protein kinases in the complex HSP90. Heat shock proteins (HSPs) are a group of important proteins *in vivo* (23). They not only help in the correct assembly, folding and transport of proteins but also participate in inhibition and apoptosis as well as correlate with antigen presentation, steroid receptor function, intracellular transport and nuclear receptor integration. Heat shock protein 90 is expressed in the plasma, nucleus and endoplasmic reticulum of the central neural system when brain injury occurs (24). It interacts temporarily with other proteins in an ATP-dependent manner to maintain the normal physiological structure and function of neurons, therefore exerting an important role in stress. Increased HSP90 ATPase activity acts on the target proteins, promoting their folding and maturation. However, whether HSP90 plays a protective role in mental stress warrants further investigation. The possible mechanisms associated with HSP90 include regulating Ca^{2+} and calmodulin, inhibiting apoptotic pathways and antagonizing the excitatory toxicity of glutamic acid. The results of this study suggest that HSP90 co-chaperone CDC37 benefits the repair to damage caused by positive stress to the brain after chronic morphine administration, which offers new insights for novel drug development and therapy for morphine dependence.

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