

## Proteome Analysis of Rat Hippocampus Following Morphine-induced Amnesia and State-dependent Learning

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### Abstract

Morphine's effects on learning and memory processes are well known to depend on synaptic plasticity in the hippocampus. Whereas the role of the hippocampus in morphine-induced amnesia and state-dependent learning is established, the biochemical and molecular mechanisms underlying these processes are poorly understood. The present study intended to investigate whether administration of morphine can change the expression level of rat hippocampal proteins during learning of a passive avoidance task. A step-through type passive avoidance task was used for the assessment of memory retention. To identify the complex pattern of protein expression induced by morphine, we compared rat hippocampal proteome either in morphine-induced amnesia or in state-dependent learning by two-dimensional gel electrophoresis and combined mass spectrometry (MS and MS/MS). Post-training administration of morphine decreased step-through latency. Pre-test administration of morphine induced state-dependent retrieval of the memory acquired under post-training morphine influence. In the hippocampus, a total of 18 proteins were identified whose MASCOT (Modular Approach to Software Construction Operation and Test) scores were inside 95% confidence level. Of these, five hippocampal proteins altered in morphine-induced amnesia and ten proteins were found to change in the hippocampus of animals that had received post-training and pre-test morphine. These proteins show known functions in cytoskeletal architecture, cell metabolism, neurotransmitter secretion and neuroprotection. The findings indicate that the effect of morphine on memory formation in passive avoidance learning has a morphological correlate on the hippocampal proteome level. In addition, our proteomic screen suggests that morphine induces memory impairment and state-dependent learning through modulating neuronal plasticity.

**Keywords:** Morphine; Hippocampus; Passive avoidance learning; Protein expression; Proteomics.

### Introduction

Morphine, a potent opiate analgesic drug, has long been used to treat severe pain. Besides

relieving pain (1), it can quickly develop tolerance and physical and psychological dependence (2). Furthermore, it has been demonstrated that morphine administration affects learning and memory processes in a dose- and time-dependent manner. For example, acute morphine administration inhibits memory

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formation in different learning paradigms such as passive avoidance learning (3,4) and spatial learning (5). Our previous studies also showed that post-training administration of morphine induces amnesia in inhibitory avoidance task (3,6,7), while pre-test administration of the same doses of morphine improves morphine-induced amnesia, showing morphine state-dependent learning (7,8). It has been very clearly demonstrated that the newly acquired information in one drug state cannot be recalled or used unless the retrieval is tested in the same drug state (9,10). In spite of several behavioral and pharmacological investigations on morphine state-dependent learning, molecular mechanisms of this phenomenon still await elucidation.

In general, the mechanisms involved in the modulation of learning and memory processes during exposure to drugs of abuse have long attracted a great deal of attention. Exposure to drugs of abuse such as nicotine, morphine and cocaine has been found to induce neuronal circuit synaptic connectivity in brain areas responsible for their rewarding properties (11). The mesocorticolimbic dopaminergic system which originates from the ventral tegmental area (VTA) and projects to the target sites including the nucleus accumbens, cortex, amygdala and hippocampus, may play a critical role in producing the reinforcing/rewarding effects of the drugs (12). Considering that hippocampal-VTA loop may regulate the entry of information into long-term memory (13), it has been suggested that there is a functional interaction between drug-induced activation of mesocorticolimbic dopaminergic reward system and the induction of hippocampal long-term potentiation (LTP) (14). It should be considered that hippocampal LTP is a critical neural process for memory formation which can be induced in inhibitory avoidance training in rats (15).

Hippocampus as a part of the limbic system is involved in processing of different types of learning (16,17). Electrophysiological studies have shown that there is a relationship between synaptic plasticity in the hippocampal pyramidal neurons and the induction of passive avoidance learning (18,19). According to molecular analyses, synaptic plasticity has two main phases: the short phase which relies on

existing proteins (20) and the long one which requires the synthesis of new proteins for *long-lasting* potentiation of synaptic transmission (21). It is important to note that proteins mediate functional and structural connectivity in neurons during learning and memory processes (22). In order to evaluate the proteins that are responsible for cognitive functions, proteomics has been used as a powerful tool which makes possible simultaneous study of a large number of proteins (23,24). Proteomic studies have shown differential protein expression during hippocampal synaptic plasticity (25,26). For example, it has been shown that the transcription factor cAMP responsive element binding protein (CREB) mediates the conversion of short term synaptic activity to long term plasticity (27).

Differential proteome expression is also associated with morphine dependence (28,29). Molecular analyses have revealed that neuronal energy metabolism, CREB phosphorylation and chaperone proteins level may alter in the hippocampus of morphine-treated rats (28,30). Using passive avoidance learning, Bianchi and colleagues (2009) (31) reported that morphine-induced amnesia increased the activation of hippocampal phospholipase C, which is a signaling effector enzyme in mediating opioid effects (32,33). Considering that morphine is used for pain relief in medicines and that it modulates learning-related plasticity, the aim of the present study was to evaluate expression changes of hippocampal proteins in trained rats treated with saline or morphine in the passive avoidance task by two-dimensional gel electrophoresis and combined mass spectrometry (MS and MS/MS). Therefore, the present work is the first study to determine whether the molecular events required for long-term memory formation of associative hippocampal-dependent tasks can be affected by acute administration of morphine.

## Experimental

### *Animals*

Male Wistar rats (Pasteur Institute; Tehran, Iran) weighing 200-230 g at the time of training were used. The animals were kept in an animal house with a 12-h light/ 12-h dark cycle and controlled temperature ( $22 \pm 2^\circ\text{C}$ ). Availability

of food and water to animals was *ad libitum*. Animals were kept in an animal room for at least 1 week to adapt to the laboratory conditions before training. All procedures were performed in accordance with institutional guidelines for animal care and use. The Research and Ethics Committee of the college of science at the University of Tehran approved the experimental protocol.

#### *Passive avoidance apparatus*

Passive avoidance apparatus for training and testing contained a box of two compartments, one light (white compartment) and the other dark (dark compartment) with the same size ( $20 \times 20 \times 30$  cm) that were connected to each other with a guillotine door ( $7 \times 9$  cm). The floor of the dark compartment was replaced with stainless steel rods (2.5 mm in diameter) at 1cm intervals to produce foot shock. Intermittent electric shocks (50 Hz, 3s, 1mA) were delivered by an insulated stimulator (Borj Sanat, Iran).

#### *Training and testing*

##### *Training*

Training was performed 30 min after habituation of the animals in the experimental room. Each animal was gently placed in the brightly lit compartment of the apparatus; the guillotine door was then opened after 5 s to let the animal enter the dark compartment. The latency with which the animal crossed into the dark compartment was recorded. Any animal waiting more than 100s in the light compartment was eliminated from the study. Once the animal entered the next compartment with all four paws, the door was closed and the animal was taken to its home cage. The trial was repeated after 30 min (acquisition trial), in which a foot shock (50 Hz, 1 mA, 3 s) was immediately delivered to the grid floor of the dark room. The rat was removed from the apparatus and placed temporarily into its home cage. Two minutes later, the procedure was repeated; if the rat did not enter the dark compartment during 120 s, it was recorded as successful acquisition of passive avoidance response. Otherwise, when the rat entered the dark compartment before 120 s, the door was closed and the same shock was applied again. Upon retesting, if the rat acquired passive

avoidance successfully, it was removed from the apparatus and put back to its home cage.

##### *Testing*

Retrieval test was performed 24 hours after training to determine long-term memory. Each animal was gently placed in the light compartment; the door was opened and the stepthrough latency was measured for entering into the dark compartment. Testing session ended when the animal entered the dark compartment or remained in the light compartment for 300 s (criterion for retrieval). During testing session, no electric shock was applied.

##### *Drugs*

The drug used in the study was morphine sulfate (Temad Co., Tehran, Iran). Morphine was dissolved in sterile 0.9% saline. Immediately after successful training, morphine was injected subcutaneously (post-training *s.c.* injection). Control animals received saline subcutaneously (*s.c.*).

#### *Experimental procedure*

##### *Effect of morphine on memory retrieval in passive avoidance task*

Three experimental groups of eight animals were used in order to evaluate the effects of post-training and/or pre-test administration of morphine on memory retrieval of passive avoidance task. One group of animals received saline (1 mL/Kg, *s.c.*) immediately after training (post-training). On the test day, the animals received saline (1 mL/Kg, *s.c.*) 30 min before the test (pre-test). Another two groups of animals received post-training injection of morphine (7.5 mg/Kg, *s.c.*) and after 23.5 h, they received pre-test injections of saline (1 mL/Kg, *s.c.*) or morphine (7.5 mg/Kg, *s.c.*). The step-through latency was measured 30 min after the injection (Figure 1).

##### *Hippocampus extraction*

In order to perform hippocampal proteomic analysis, three animals with the best step-through latency score were randomly chosen from each group. Each animal was sacrificed by rapid guillotine decapitation after the test session. The brain was removed from the skull and the

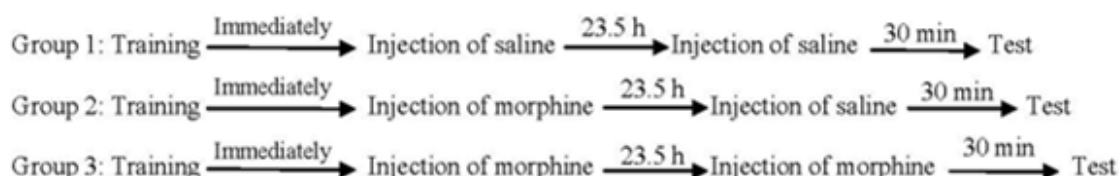


Figure 1. Summary of experimental design in passive avoidance task.

hippocampus extracted quickly and easily from rats as described by Chiu and co-workers (2007) (34). The hippocampus was frozen in liquid nitrogen before storing at  $-80^{\circ}\text{C}$ .

#### Protein extraction

Protein extraction was performed as described by Hirano *et al.* (35) with some modifications. Trichloroacetic acid (TCA)/acetone extraction buffer (AEB) was used for protein extraction. TCA-AEB is a suitable solution for protein extraction which denatures proteins and inactivates enzymes (see Table 1). AEB is used to eliminate the interference effect of TCA in isoelectric focusing (IEF). It should be considered that one of the most important considerations in two-dimensional gel electrophoresis (2DE) is the preparation of protein samples. All the buffers and detergents should be compatible with IEF. The choice of material for protein extraction requires considerations of salt concentration and protein solubility, because high concentration of salt and protein precipitation can be problematic. Among protocols, TCA-AEB is the most suitable extraction method for reliable IEF. Each hippocampus was homogenized separately

with pestle in TCA/AEB, and centrifuged at 15000 rpm, 15 min, at  $4^{\circ}\text{C}$  after incubation for 1 h at  $-20^{\circ}\text{C}$ . Lysis buffer (7M Urea, 2M thiourea, 4% CHAPS, 18 mM Tris-HCL (PH 8.0), 50 mM DTT) was added to the pellet and centrifuged at 15000 rpm for 20 min at  $10^{\circ}\text{C}$ . The protein concentration of the supernatant was estimated (20-30  $\mu\text{g}/\mu\text{L}$ ) through Bradford method (36) using NanoDrop 2000c Thermo Spectrophotometer.

#### Two-dimensional gel electrophoresis

The first dimension started with Rehydration step in which a Rehydration buffer (8 M urea, 4 % CHAPS, 0.2 % ampholyte pH 3-10, 50 mM DTT ) containing 1mg protein sample was applied onto Non-linear immobilized pH gradient strips (NL IPG, pH 3-10, 17cm). Isoelectric focusing (IEF) was carried out with Protean IEF Cell (BioRad, USA) at 50000 Vh. Before starting the second dimension, Equilibration buffer (6 M Urea, 2% SDS, 50 mM Tris-HCL (pH 8.8), 20% v/v Glycerol) containing 2% DTT (first step) or 2.5% Iodoacetamide (second step) was applied onto focused strips for 20 min. The second dimension was carried out according to Laemmli

Table 1. Protein extraction solutions.

Protein extraction solution	Amount	Material
TCA-AEB	10%	trichloroacetic acid (TCA)
	0.07%	Merchптоethanol
	To needed volume	Acetone
AEB	0.07%	Merchптоethanol
	To needed volume	Acetone
Lysis buffer	7M	Urea
	2M	Thiourea
	4% (v/v)	CHAPS
	18 mM	Tris-HCL (PH 8.0)
	50 mM	Dithioeritol (DTT)
	To needed volume	Ultra pure water

buffer system (37), using handmade 12% SDS-polyacrylamide gels and run in Protean II xi Cell (BioRad, USA). Each gel was placed separately in bluesilver staining solution for one night (38).

#### *Image analysis*

Analytical gels were scanned by a Densitometer GS-800 (BioRad) scanner at 300 dpi in tagged image file format (TIFF). ImageMaster™ 2D Platinum v6.0 software was then used to extract and digitize data from graphical images of scanned gels through detecting, normalizing, matching and comparing protein spots according to their volume percent. Student t-test was performed and spots with more than 1.5 fold changes in vol% selected for identification by mass spectrometry and statistical significance was  $p < 0.05$ .

#### *In-gel trypsin digestion of proteins*

Spots were manually cut from 2-DE gels, placed in 96 wells v-shape polypropylene plates and dried completely. The Ettan Spot Handling Workstation (GE Healthcare, UK) was used for automatic in-gel digestion of samples. Each gel plug was soaked in 100  $\mu$ L of washing solution (50% MeOH, 50 mM  $\text{NH}_4\text{HCO}_3$ ) to re-swell and was then washed two more times in the same solution. The gel plugs were further washed twice in 75% ACN, before being completely dried. Samples were then re-hydrated by adding freshly prepared trypsin solution (0.5  $\mu$ g modified porcine trypsin in 25  $\mu$ L 20 mM  $\text{NH}_4\text{HCO}_3$ ), and were incubated for 240 min at 37 °C. Peptides were extracted from the gel plugs, by washing twice in 100  $\mu$ L of 50% ACN, 0.1% TFA and transferred in solution to a fresh 96 well plate, where samples were dried.

#### *Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF/TOF-MS)*

Tryptic peptides were resuspended in 3  $\mu$ L of 50% ACN, 0.1%TFA. 0.3  $\mu$ L of resuspended tryptic peptides were spotted onto a steel Applied Biosystems 192 sample MALDI target plate, and were mixed (while wet) with 0.3  $\mu$ L of a 90% saturated  $\mu$ -cyano-4-hydroxycinnamic acid (CHCA) in 50% ACN, 0.1% TFA. The dried samples were analyzed using a MALDI-

TOF/TOF MS (4700 Proteomics Analyzers, Applied Biosystems, UK), performing MS analysis and subsequent MS/MS analysis on up to 10 precursor peptides. Each sample was internally calibrated by reference to specific autolytic fragments of trypsin. Mass tolerance settings of 1.2 Da for parent ion and 0.5 Da for fragment ions were applied. Search settings allowed one missed cleavage with trypsin and two modifications (carboxamidomethylation of cysteine and oxidation of methionine). The PMF and MS/MS information were automatically searched against the NCBI non-redundant database using the Mascot search engine (Matrix Science, UK). The Mascot score cut-off value was set to 88 for the statistical confidence limits of 95%.

#### *Statistical analysis of memory retrieval*

The step-trough latencies as an index of memory retrieval (Figure 2) are expressed as means  $\pm$  S.E.M. The statistical analysis was performed using one-way analysis of variance (ANOVA). The level of statistical significance was set at  $p < 0.05$ . Post-hoc comparison of means was carried out using Tukey test for comparisons. Calculations were performed using the SPSS statistical package (Version 20).

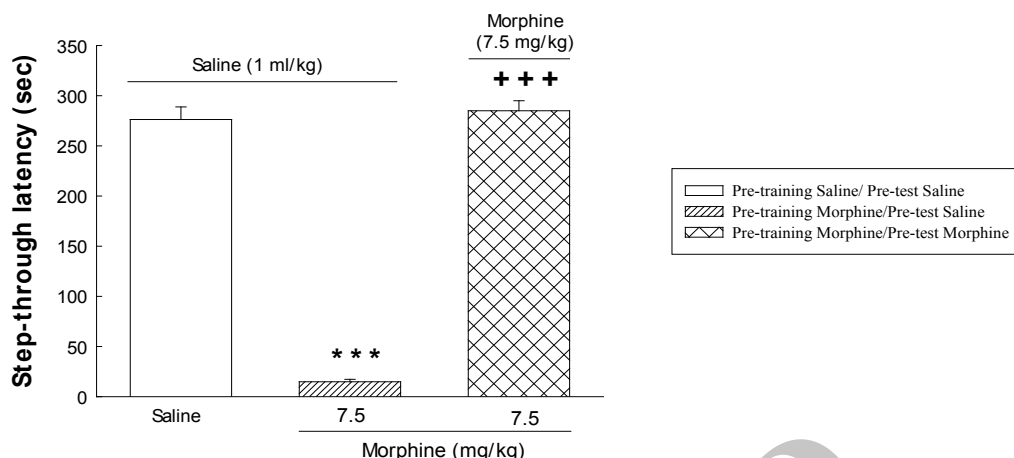
## **Results**

#### *Effects of morphine on memory retrieval*

Figure 1 shows the effect of morphine on memory retrieval in passive avoidance task. One-way ANOVA revealed that morphine administration altered memory retrieval [ $F(2, 21)=269.0, P<0.001$ ]. Further analysis showed that post-training administration of morphine (7.5 mg/Kg, s.c.) impaired memory retrieval, indicating an amnesic effect. Pre-test administration of the opioid restored the impairment induced by post-training injection of the drug. Post hoc analysis showed that Maximum restoration was obtained with 7.5 mg/Kg of morphine.

#### *Identification of differentially expressed proteins*

Comparison of the protein profiles in the control group, the amnesia group and morphine



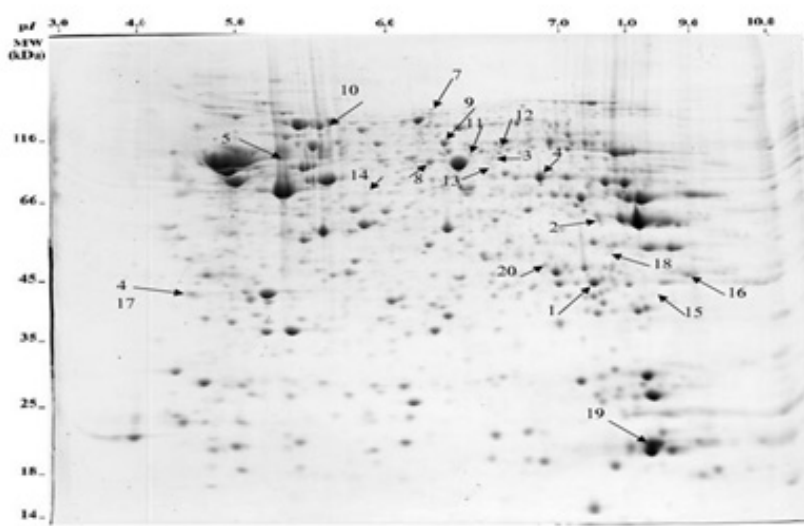
**Figure 2.** The effect of post-training administration of morphine on step-through latencies. The animals received post-training saline (1 mL/Kg, *s.c.*) or morphine (7.5 mg/Kg) and were tested after 24 h. Each value represents the mean  $\pm$  S.E.M. of eight rats per group. \*\*\* $P$  < 0.001 compared with the saline/saline group.+++ $P$  < 0.001 compared with the morphine/saline group.

state-dependent learning group showed different protein expression patterns which are mapped in Figure 2. Protein identification was performed by peptide mass fingerprint (PMF) and tandem mass spectrometry (MS/MS) analysis on up to 10 precursor peptides. Detailed characteristics of the identified proteins resulted from MALDI-TOF MS and MASCOT search engine are presented in Table 2. Statistical confidence limits of 95% were

applied for protein identification.

### Discussion

Morphine which is used for pre and post operative pain management also modulates hippocampal-related learning (7), predominantly through  $\mu$ -opioid receptors in a time- and dose-dependent manner. Our previous studies had shown that morphine could induce state-



**Figure 3.** A representation of hippocampus proteome profile following morphine administration. Protein extracts are separated according to their molecular weight at the second dimension using 12% SDS-PAGE. Arrowheads show different expressed proteins.

**Table 2.** Summary of the identified proteins differentially expressed among the three different groups of rats.

Identified proteins from comparison of the amnesia and the control groups.							
Rank	Protein name	Acces. No.	Protein MW	Protein pI	Peptide count	Protein score	Relative protein content
1	rCG29914, isoform CRA-b	gi 149049470	31675.8	5.56	13	195	Down-regulation (1.6 folds)
2	Coagulation factor 5/8 type domain protein	gi 150386371	145370.2	8.91	20	88	Down-regulation (1.5 folds)
3	Unnamed protein product	gi 56107	47428.3	6.16	13	145	Down-regulation (2 folds)
	<b>Protein group</b> Enolase 1, (alpha) isoform1	gi 158186649	47440.4	6.16			
4	RecName: Full=Synaptosomal-associated protein 25-B; Short=SNAP-B; AltName: Full Synaptosome-associated protein 25.2; Short=SNAP-25.2	gi 82202584	22906.8	4.46	10	102	Up-regulation (2.1 folds)
5	RecName: Full=Tubulin beta-1 chain; AltName: Full=Beta-tubulin class-I	gi 135446	50333.1	4.78	13	145	Up-regulation (2.4 folds)
	<b>Protein group</b> Class II beta tubulin isotype	gi 27227551	50283.1	4.82			
	Tubulin T beta15	gi 224839	50361.1	4.79			
	Tubulin, beta2	gi 4507729	50274.1	4.78			
Identified proteins from comparison of morphine dependent learning group and the control group							
Rank	Protein name	Acces.No	Protein MW	Protein pI	Peptide count	Protein score	Relative protein content
6	Glul protein	gi 38181948	42982.6	6.47	16	201	Up-regulation (2 folds)
	<b>Protein group</b> Glutamine synthetase	gi 204349	43000.6	6.83			
	Glutamine synthetase1	gi 142349612	42981.6	6.64			
7	Ezrin	gi 52138521	69461.7	5.83	19	144	Up-regulation (1.9 folds)
8	Unnamed protein product	gi 56107	47428.3	6.16			Up-regulation (1.6 folds)
	<b>Protein group</b> Enolase 1, (alpha) isoform1	gi 158186649	47440.4	6.16			
9	Coronin, actin binding protein 1A	gi 18426834	51717.2	6.05	92	99.343	Up-regulation (1.9 folds)
10	Serine/threonine-protein kinase 9/CDK15	gi 239881816	61927.3	9.39	15	90	Down-regulation (1.7 folds)
11	rCG53214, isoform CRA_d	gi 149060018	49147	6.23	13	88	Down-regulation (1.5 folds)
12	rCG55098 protein group	gi 149045822	58135.6	6.62	13	129	Down-regulation (1.5 folds)
	aldehyde dehydrogenase 1B1	gi 58865518	58101.6	6.62			
13	Peroxioredoxin 5, isoform CRA_b	gi 149062197	18966.9	9.22	10	99	Down-regulation (1.6 folds)
14	ATPase, H transporting, lysosomal V1 subunit F	gi 16758754	13362	5.52	9	120	Down-regulation (1.5 folds)

**Table 2.** (Continued).

Identified proteins from comparison of morphine dependent learning group and the amnesia group

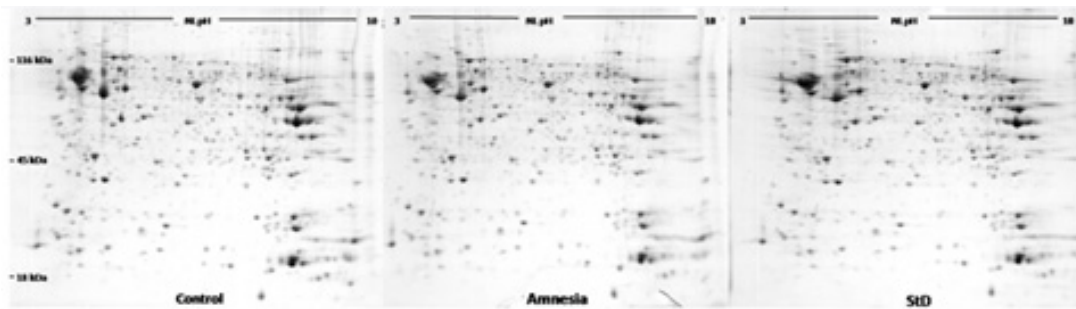
Rank	Protein name	Acces. No	Protein MW	Protein pI	Peptide count	Protein score	Relative protein content
15	Hypothetical protein	gi 169774989	84365.7	5.53	16	82	Detected only in morphine state dependent learning
16	Succinate dehydrogenase complex, subunit B, iron sulfur (Ip) (predicted), isoform CRA_f	gi 149024459	24745.1	8.38	12	122	Down-regulation (1.6 folds)
17	RecName: Full=Synaptosomal-associated protein 25-B; Short=SNAP-B; AltName: Full Synaptosome-associated protein 25.2; Short=SNAP-25.2	gi 82202584	22906.8	4.46	10	102	Down-regulation (2.3 folds)
18	Carbonyl reductase 1	gi 76779821	30817	8.22	10	83	Down-regulation (1.6 folds)
19	Hemoglobin beta chain complex	gi 17985949	16083.3	7.88	12	347	Up-regulation (1.7 folds)
20	Pyridoxine 5'-phosphate oxidase	gi 12018270	30507.1	8.66	10	94	Up-regulation (1.7 folds)

dependent learning (StD) which is a dual action of the opiate on learning and memory processes (3). Using passive avoidance learning, our previous results also showed that pre- or post-training administration of morphine induced amnesia via inhibiting the acquisition or consolidation of memory while pre-test administration of the same doses of the drug improved amnesia through facilitating memory retrieval (6, 7). Considering that morphine administration alters the proteome profile of the hippocampus, we hypothesized that differential expression of hippocampal proteins may be involved in morphine-induced amnesia and state-dependent learning. It should be considered that neuroproteomics have been widely used to evaluate brain signal complexity and also help diagnose neuronal disorders. For example, the detailed proteome database of hippocampus reported by Fountoulakis and colleagues (2005) may be useful to better understanding of memory formation, and disorders like dementias, recurrent major depression and Cushing's disease (39). In addition, a few studies, which have focused on neuroadaptive mechanisms associated with morphine dependence, have reported several profound changes in rat brain proteome following chronic treatment (28,29).

On the basis of the above findings, in the present study, 2DGE (Figure 3) was used to detect differentially expressed proteins in two groups of animals which showed the dual action of morphine on passive avoidance learning compared with a control group. Three gels per group were prepared and comparison of protein spot patterns of gels across all groups was made by calculating the coefficients of variation of normalized spot volumes. Differentially expressed spots were excised for identification of proteins by combined MALDI-TOF-MS/MS. Finally 18 proteins whose MASCOT scores were inside 95% confidence level were obtained. The detailed properties of the proteins are listed in Table 2. These proteins belong to different functional groups including cytoskeleton, neurotransmitter secretion, energy metabolism and oxidative damage protection.

The present results showed that five hippocampal proteins altered in morphine-induced amnesia. It was found that post-training administration of morphine increased SNAP-25 protein level. SNAP-25 is one of the SNARE proteins which mediate presynaptic vesicular docking processes in exocytosis and neurotransmission release (40, 41). This protein exists predominantly in hippocampal





**Figure 3.** Three representative images of blue silver staining 2DE gels from hippocampal samples of control group, amnesia group and morphine state-dependent learning group. Proteins were separated on 18-cm IPG strips with nonlinear (NL) pH 3–10 in the first dimension and on SDS-polyacrylamide gel (12%) in the second dimension.

glutamatergic synapses (42), which is the most important excitatory synapses during hippocampal-related learning processes such as passive avoidance learning consolidation (43,44). SNAP-25 has also been shown to regulate neuronal excitability via negatively regulating calcium responsiveness in glutamatergic neurons (42). It has been suggested that down-regulation of SNAP-25 after passive avoidance training (45), increases neuronal excitability required for memory consolidation (46). In our study, down-regulation of SNAP-25 suggest that amnesia, which was induced by post-training administration of morphine, may be partially due to decrease in neuronal excitability. An unnamed protein product which belongs to the group of enolase 1 was down regulated. It is well known that enolase enzymes have an important role in glycolysis and gluconeogenesis (47). Inhibitory effect of morphine on glycolytic enzymes has previously been observed (48,49). It is suggested that morphine-induced glucose hypometabolism may result in hippocampal memory impairment (50). It should however be noted that neuron specific enolase has been used as a marker of cognitive disorder (51, 52). In addition to glucose metabolism,  $\alpha$ -enolase also participates in a variety of cellular regulatory processes (53). The obtained results also indicated that post-training administration of morphine up-regulated  $\alpha$ -tubulin level.  $\alpha$ -tubulin is the structural unit of microtubules which mediate the organization of neuronal axons and dendrites in memory-related synaptic plasticity (54). It has been previously shown that the induction of

microtubule depolymerization causes amnesia (55, 56), Up-regulation of  $\alpha$ -tubulin observed in the present study may therefore be due to the depolymerization of microtubules in morphine-induced amnesia.

Also, in the present study, ten proteins were found to change in the hippocampus of animals that received post-training and pre-test morphine. Interestingly, the majority of changes of hippocampal protein expression have been observed in morphine-induced state-dependent learning. These proteins are functionally divided into the groups of cytoskeleton (Ezrin, Coronin 1A), neurotransmitter secretion (Glut protein, ATPase H transporting lysosomal V1 subunit F) and neuroprotection (peroxiredoxin 5 and rCG55098). Ezrin and coronin 1A are two F-actin binding proteins which exhibit upregulation in morphine-induced StD. Ezrin is one of the ERM (ezrin, radixin and moesin) proteins in peripheral astrocyte processes (PAPs) (57), which mediate positive effects on memory-related synaptic plasticity (58, 59). The other cytoskeletal-related protein, coronin 1A, belongs to the type I coronins (60), which mediate the rearrangement of membrane cytoskeleton and may be involved in intracellular membrane transport (61). It has been reported that coronin 1A expression decreases in the hippocampus of aged animals which is indicative of cognitive decline and synaptic loss in the hippocampus (62). Furthermore, glutamine synthetase (Glut protein) is a neurotransmission-related protein which exhibits up-regulation in morphine-induced state-dependency. Glutamine synthetase is an important enzyme which removes excess

cerebral glutamate through glutamate–glutamine cycle to avoid neurotoxicity effects of glutamate. Inhibition of Glut protein activity increases extracellular concentration of glutamate (63,64) which results in the impairment of long-term potentiation and cognitive dysfunction (65,66). According to data mentioned above, it can be suggested that hippocampal glutamate neurotransmission may have an important role in morphine state-dependent learning.

On the other hand, a comparative proteome analysis of the hippocampus from two groups of rats that received post-training administration of morphine and pre-test administration of saline (the amnesia group) or morphine (the StD group) showed that hemoglobin  $\beta$ -chain protein expression was up-regulated in the hippocampus of the StD group. Hemoglobin gene expression has been reported in different parts of the brain including the hippocampus (67). It should be considered that neuronal hemoglobin chains are sources of hemorphines which bind to opioid receptors and participate in memory retention and retrieval (68). Furthermore, we found that pyridoxine 5'-phosphate oxidase (PNPO) protein level was up-regulated in morphine dependent leaning group. It is suggested that PNPO up-regulation may increase neuronal excitability and induce long term potentiation in the hippocampus (69,70). Our proteomic approach revealed that morphine administration down-regulated protective enzymes against oxidative damages including Peroxiredoxin 5, carbonyl reductase 1 and rCG55098. Moreover, similar to previous studies (48,49), morphine administration altered the expression of proteins involved in energy metabolism including succinate dehydrogenase complex subunit B and an unnamed protein belonging to enolase 1 group.

The present study offered the first proteome profile of the rat hippocampus in acute morphine administration. Our results revealed differential expression of proteins in morphine-induced amnesia and state-dependent learning. The identified proteins belong to different functional groups including cytoskeleton, neurotransmission, energy metabolism and neuroprotection. Most of the identified proteins including SNAP-25, Glutamine synthetase,  $\alpha$ -tubulin, ezrin, Coronin

1A and pyridoxine 5'-phosphate oxidase are involved directly or indirectly in synaptic-related processes. Therefore, our proteomic screen suggests that morphine induces memory impairment and state-dependent learning through modulating neuronal plasticity. Our results also showed that a single exposure to morphine can produce prolonged effects on the hippocampal signaling cascades required for long-term memory formation. However, additional assessments are required to further evaluate the outcome of 2 DGE- MALDI-TOF MS method.

## References

- (1) Akbari E, Mirzaei E and Shahabi Majd N. Long-term morphine-treated rats are more sensitive to antinociceptive effect of diclofenac than the morphine-naive rats. *Iran. J. Pharm. Res.* (2013) 12: 175-184.
- (2) Bodnar RJ. Endogenous opiates and behavior: 2010. *Peptides* (2011) 32: 2522-2552.
- (3) Rassouli Y, Rezayof A and Zarrindast MR. Role of the central amygdala GABA-A receptors in morphine state-dependent memory. *Life Sci.* (2010) 86: 887-893.
- (4) Galeotti N, Ghelardini C and Bartolini A. Differential prevention of morphine amnesia by antisense oligodeoxynucleotides directed against various Gi-protein alpha subunits. *Br. J. Pharmacol.* (2001) 133: 267-274.
- (5) Ma MX, Chen YM, He J, Zeng T and Wang JH. Effects of morphine and its withdrawal on Y-maze spatial recognition memory in mice. *Neurosci.* (2007) 147: 1059-1065.
- (6) Darbandi N, Rezayof A and Zarrindast MR. Modulation of morphine state-dependent learning by muscarinic cholinergic receptors of the ventral tegmental area. *Physiol. Behav.* (2008) 94: 604-610.
- (7) Khajehpour L, Rezayof A and Zarrindast MR. Involvement of dorsal hippocampal nicotinic receptors in the effect of morphine on memory retrieval in passive avoidance task. *Eur. J. Pharmacol.* (2008) 584: 343-351.
- (8) Zarrindast MR and Rezayof A. Morphine state-dependent learning: sensitization and interactions with dopamine receptors. *Eur. J. Pharmacol.* (2004) 497: 197-204.
- (9) Overton DA. Historical context of state dependent learning and discriminative drug effects. *Behav. Pharmacol.* (1991) 2: 253-264.
- (10) Shulz DE, Sosnik R, Ego V, Haidarliu S and Ahissar E. A neuronal analogue of state-dependent learning. *Nature* (2000) 403: 549-553.
- (11) Robinson TE and Kolb B. Structural plasticity associated with exposure to drugs of abuse. *Neuropharmacol.* (2004) 47: 33-46.
- (12) Tzschentke TM. Measuring reward with the

- conditioned place preference (CPP) paradigm: update of the last decade. *Addict. Biol.* (2007) 12: 227-462.
- (13) Lisman JE and Grace AA. The hippocampal-vta loop: controlling the entry of information into long-term memory. *Neuron.* (2005) 46: 703-713.
- (14) Wittmann BC, Schott BH, Guderian S, Frey JU, Heinze HJ and Düzel E. Reward-related fMRI activation of dopaminergic midbrain is associated with enhanced hippocampus-dependent Long-Term Memory Formation. *Neuron.* (2005) 45: 459-467.
- (15) Whitlock JR, Heynen AJ, Shuler MG and Bear MF. Learning induces long-term potentiation in the hippocampus. *Sci.* (2006) 313: 1093-1097.
- (16) Morris RGM. Elements of a neurobiological theory of hippocampal function: the role of synaptic plasticity, synaptic tagging and schemas. *Eur. J. Neurosci.* (2006) 23: 2829-2846.
- (17) Assadiannarejji S, Naghdi N, Oryan S and Azadmanesh K. Intrahippocampal Injection of 3 $\alpha$  Diol (a Testosterone Metabolite ) and Indomethacin (3 $\alpha$ -HSD Blocker), impair acquisition of spatial learning and memory in adult male rats. *Iran. J. Pharm. Res.* (2013) 12: 457-469
- (18) Kudryashova IV. Responses of hippocampal neurons at different stages. *Neurosci. Behav. Physiol.* (2002) 32: 121-127.
- (19) Lashgari R, Motamedi F, Zahedi Asl S, Shahidi S and Komaki A. Behavioral and electrophysiological studies of chronic oral administration of L-type calcium channel blocker verapamil on learning and memory in rats. *Behav. Brain Res.* (2006) 171: 324-328.
- (20) Soderling TR and Derkach VA. Postsynaptic protein phosphorylation and LTP. *Trends Neurosci.* (2000) 23: 75-80.
- (21) Stough S, Shobe JL and Carew TJ. Intermediate-term processes in memory formation. *Curr. Opin. Neurobiol.* (2006) 16: 672-678.
- (22) Cajigas IJ, Will T and Schuman EM. Protein homeostasis and synaptic plasticity. *EMBO J.* (2010) 29: 2746-2752.
- (23) Fountoulakis M. Application of proteomics technologies in the investigation of the brain. *Mass Spectrom. Rev.* (2004) 23: 231-258.
- (24) Marcus K, Schmidt O, Schaefer H, Hamacher M, van Hall A and Meyer HE. Proteomics application to the brain. *Int. Rev. Neurobiol.* (2004) 61: 285-311.
- (25) McNair K, Davies CH and Cobb SR. Plasticity-related regulation of the hippocampal proteome. *Eur. J. Neurosci.* (2006) 23: 575-580.
- (26) Zheng JF, Patil SS, Chen WQ, An W, He JQ, Höger H and Lubec G. Hippocampal protein levels related to spatial memory are different in the barnes maze and in the multiple t-maze. *Proteome Res.* (2009) 8: 4479-4486.
- (27) Sakamoto K, Karelina K and Obrietan K. CREB: a multifaceted regulator of neuronal plasticity and protection. *J. Neurochem.* (2011) 116: 1-9.
- (28) Bierczynska-Krzysik A, Bonar E, Drabik E, Noga M, Suder P, Dylag T and Dubin A. Rat brain proteome in morphine dependence. *Neurochem. Int.* (2006) 49: 401-406.
- (29) Kim SY, Chudapongse N, Lee SM, Levin MC, Oh JT, Park HJ and Ho IK. Proteomic analysis of phosphotyrosyl proteins in morphine-dependent rat brains. *Mol. Brain Res.* (2005) 133: 58-70.
- (30) Yang HY and Pu XP. Chronic morphine administration induces over-expression of aldolase C with reduction of CREB phosphorylation in the mouse hippocampus. *Eur. J. Pharmacol.* (2009) 609: 51-57.
- (31) Bianchi E, Lehmann D, Vivoli E, Norcini M and Ghelardini C. Involvement of PLC- $\beta$ 3 in the effect of morphine on memory retrieval in passive avoidance task. *Psychopharmacol.* (2009)24: 891-896.
- (32) Galeotti N, Stefano GB, Guarna M, Bianchi E and Ghelardini C. Signaling pathway of morphine induced acute thermal hyperalgesia in mice. *Pain.* (2006) 123: 294-305.
- (33) Liu NJ, Gizycki H and Gintzler AR. Phospholipase C $\beta$ 1 modulates pain sensitivity, opioid antinociception and opioid tolerance formation. *Brain Res.* (2006) 1069: 47-53.
- (34) Chiu K, Lau WM, Lau HT, So K and Chang RC. Microdissection of rat brain for rna or protein extraction from specific brain region. *J. Vis. Exp.* (2007) 7: 269.
- (35) Hirano M, Rakwal R, Shibato J, Agrawal GK, Jwa NS, Iwahashi H and Masuo Y. New protein extraction/ solubilization protocol for gel-based proteomics of rat (female) whole brain and brain regions. *Mol. Cells* (2006) 22: 119-125.
- (36) Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* (1976) 72: 248-254.
- (37) Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* (1970) 227: 680-685.
- (38) Candiano G, Bruschi M, Musante L, Santucci L, Ghiggeri GM, Carnemolla B, Orecchia P, Zardi L and Righetti PG. Blue silver: A very sensitive colloidal Coomassie G-250 staining for proteome analysis. *Electrophoresis* (2004) 25: 1327-1333.
- (39) Fountoulakis M, Tsangaris GT, Maris A and Lubec G. The rat brain hippocampus proteome. *J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci.* (2005) 819: 115-129.
- (40) Sollner T, Bennett MK, Whiteheart SW, Scheller RH and Rothman JE. A protein assembly-disassembly pathway *in-vitro* that may correspond to sequential steps of synaptic vesicle docking, activation, and fusion. *Cell* (1993) 75: 409-418.
- (41) Jena BP. Membrane fusion: role of SNAREs and calcium. *Protein Pept. Lett.* (2009) 16: 712-717.
- (42) Verderio C, Pozzi D, Pravettoni E, Inverardi F, Schenk U, Coco S, Proux-Gillardeaux V, Galli T, Rossetto O, Frassoni C and Matteoli M. SNAP-25 modulation of calcium dynamics underlies differences in GABAergic and glutamatergic responsiveness to depolarization. *Neuron.* (2004) 41: 599-610.
- (43) Elvander-Tottie E, Eriksson TM, Sandin J and Ogren SO. 5-HT (1A) and NMDA receptors interact in the rat

- medial septum and modulate hippocampal-dependent spatial learning. *Hippocampus* (2009) 19: 1187-1198.
- (44) Morgado-Bernal I. Learning and memory consolidation: linking molecular and behavioral data. *Neurosci.* (2011) 176: 12-19
- (45) O'Sullivan NC, Mc Gettigan PA, Sheridan GK, Pickering M, Conboy L, O'Connor JJ, Moynagh PN, Higgins DG, Regan CM and Murphy KJ. Temporal change in gene expression in the rat dentate gyrus following passive avoidance learning. *J. Neurochem.* (2007) 101: 1085-1098.
- (46) McKee AG, Loscher JS, O' Sullivan NC, Chadderton N, Palfi A, Batti L, Sheridan GK, O'Shea S, Moran M, McCabe O, Fernández AB, Pangalos MN, O'Connor JJ, Regan CM, O'Connor WT, Humphries P, Farrar GJ and Murphy KJ. AAV-mediated chronic over-expression of SNAP-25 in adult rat dorsal hippocampus impairs memory-associated synaptic plasticity. *Neurochem.* (2010) 112: 991-1004.
- (47) Keller A, Berod A, Dussaillant M, Lamande N, Gros F and Lucas M. Coexpression of alpha and gamma enolase genes in neurons of adult rat brain. *J. Neurosci. Res.* (1994) 38: 493-504.
- (48) Sherman AD and Mitchell CL. Effects of morphine and pain on brain intermediary metabolism. *Neuropharmacol.* (1972) 11: 871-877.
- (49) Dodge PW and Takemori AE. Effects of morphine, nalorphine and pentobarbital alone and combination on cerebral glycolytic substrates and cofactors of rats *in-vivo*. *Biochem. Pharmacol.* (1972) 21: 287-294.
- (50) Chen XL, Lu G, Gong YX, Zhao LC, Chen J and Chi ZQ. Expression changes of hippocampal energy metabolism enzymes contribute to behavioural abnormalities during chronic morphine treatment. *Cell Res.* (2007) 17: 689-700.
- (51) el-Mallakh RS, Egan M and Wyatt RJ. Creatine kinase and enolase: intracellular enzymes serving as markers of central nervous system damage in neuropsychiatric disorders. *Psychiatry* (1992) 55: 392-402.
- (52) Palumbo B, Siepi D, Sabalich I, Tranfaglia C and Parnetti L. Cerebrospinal fluid neuron-specific enolase: a further marker of Alzheimer's disease? *Funct. Neurol.* (2008) 23: 93-96.
- (53) Butterfield DA and Bader Lange ML. Multifunctional roles of enolase in Alzheimer's disease brain: beyond altered glucose metabolism. *J. Neurochem.* (2009) 111: 915-933.
- (54) Dent EW, Merriam EB and Hu X. The dynamic cytoskeleton: backbone of dendritic spine plasticity. *Curr. Opin. Neurobiol.* (2010) 21: 1-7.
- (55) Nakayama T and Sawada T. Involvement of microtubule integrity in memory impairment caused by colchicines. *Pharmacol. Biochem. Behav.* (2002) 71: 119-138.
- (56) Mitsuyama F, Futatsugi Y, Okuya M, Karagiozov K, Kato Y, Kanno T, Sano H and Koide T. Microtubules to form memory. *Ital. J. Anat. Embryol.* (2004) 113: 227-235.
- (57) Derouiche A and Frotsher M. Peripheral astrocyte processes: monitoring by selective immunostaining for the actin-binding ERM proteins. *Glia.* (2001) 36: 330-341.
- (58) Derouiche A, Anlauf E, Aumann G, Mühlstädt B and Laviell M. Anatomical aspects of glia-synapse interaction: the perisynaptic glial sheath consists of a specialized astrocyte compartment. *J. Physiol. Paris.* (2002) 96: 177-182.
- (59) Cornell-Bell AH, Prem GT and Smith SJ. The excitatory neurotransmitter glutamate causes filopodia formation in cultured hippocampal astrocytes. *Glia.* (1990) 3: 322-334.
- (60) Utrecht AC and Bear JE. Coronins: the return of the crown. *Trends Cell Biol.* (2006) 16: 421-426.
- (61) Rybakina V and Clemen CS. Coronin proteins as multifunctional regulators of the cytoskeleton and membrane trafficking. *Bio. Essays* (2005) 27: 625-632.
- (62) Freeman WM, Vanguilder HD, Bennett C and Sonntag WE. Cognitive performance and age-related changes in the hippocampal proteome. *Neurosci.* (2009) 159: 183-195.
- (63) Jabaudon D, Shimamoto K, Yasuda-Kamatani Y, Scanziani M, Gähwiler BH and Gerber U. Inhibition of uptake unmasks rapid extracellular turnover of glutamate of nonvesicular origin. *Proc. Natl. Acad. Sci. USA* (1999) 96: 8733-8738.
- (64) Ni Y and Parpura V. Dual regulation of Ca<sup>2+</sup>-dependent glutamate release from astrocytes: Vesicular glutamate transporters and cytosolic glutamate levels. *Glia* (2009) 57: 1296-1305.
- (65) Katagiri H, Tanaka K and Manabe T. Requirement of appropriate glutamate concentrations in the synaptic cleft for hippocampal LTP induction. *Eur. J. Neurosci.* (2001) 14: 547-553.
- (66) Zeng LH, Ouyang Y, Gazit V, Cirrito JR, Jansen LA, Ess KC, Yamada KA, Wozniak DF, Holtzman DM, Gutmann DH and Wong M. Abnormal glutamate homeostasis and impaired synaptic plasticity and learning in a mouse model of tuberous sclerosis complex. *Neurobiol. Dis.* (2007) 28: 184-196.
- (67) Richter F, Meurers B H, Zhu C, Medvedeva VP and Chesselet MF. Neurons express hemoglobin and chains in rat and human brains. *Comp. Neurol.* (2009) 515: 538-547.
- (68) Nyberg F, Sanderson K and Glamsta EL. The hemorphins: a new class of opioid peptides derived from the blood protein hemoglobin. *Biopolymers* (1997) 43: 147-156.
- (69) Kwak SE, Kim JE, Kim DW, Kwon OS, Choi SY and Kang TC. Enhanced pyridoxal 5'-phosphate synthetic enzyme immunoreactivities do not contribute to GABAergic inhibition in the rat hippocampus following pilocarpine-induced status epilepticus. *Neurosci.* (2009) 159: 1108-1118.
- (70) Kwak SE, Kim JE, Kim DW, Kwon OS, Choi SY and Kang TC. Pyridoxine 5'-phosphate oxidase, not Pyridoxal kinase, involves in long-term potentiation induction in the rat dentate gyrus. *Hippocampus* (2009) 19: 45-56.