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# Expression changes of hippocampal energy metabolism enzymes contribute to behavioural abnormalities during chronic morphine treatment

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Dependence and impairment of learning and memory are two well-established features caused by abused drugs such as opioids. The hippocampus is an important region associated with both drug dependence and learning and memory. However, the molecular events in hippocampus following exposure to abused drugs such as opioids are not well understood. Here we examined the effect of chronic morphine treatment on hippocampal protein expression by proteomic analyses. We found that chronic exposure of mice to morphine for 10 days produced robust morphine withdrawal jumping and memory impairment, and also resulted in a significant downregulation of hippocampal protein levels of three metabolic enzymes, including Fe-S protein 1 of NADH dehydrogenase, dihydrolipoamide acetyltransferase or E2 component of the pyruvate dehydrogenase complex and lactate dehydrogenase 2. Further real-time quantitative PCR analyses confirmed that the levels of the corresponding mRNAs were also remarkably reduced. Consistent with these findings, lower ATP levels and an impaired ability to convert glucose into ATP were also observed in the hippocampus of chronically treated mice. Opioid antagonist naltrexone administrated concomitantly with morphine significantly suppressed morphine withdrawal jumping and reversed the downregulation of these proteins. Acute exposure to morphine also produced robust morphine withdrawal jumping and significant memory impairment, but failed to decrease the expression of these three proteins. Intrahippocampal injection of D-glucose before morphine administration significantly enhanced ATP levels and suppressed morphine withdrawal jumping and memory impairment in acute morphine-treated but not in chronic morphine-treated mice. Intraperitoneal injection of high dose of D-glucose shows a similar effect on morphine-induced withdrawal jumping as the central treatment. Taken together, our results suggest that reduced expression of the three metabolic enzymes in the hippocampus as a result of chronic morphine treatment contributes to the development of drug-induced symptoms such as morphine withdrawal jumping and memory impairment.

Keywords: hippocampus, glucose, morphine, dependence, learning and memory

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# Introduction

The two well-established adverse effects of opioids are dependence and impairment of learning and memory. Dependence is manifested by a withdrawal syndrome

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typically observed after the cessation of drug administration or precipitation with an antagonist such as naloxone (physical dependence), and by a compulsive drug seeking and taking behaviour (psychological dependence) [1]. Both acute and chronic exposures to morphine lead to physical dependence [2-4]. Impairment of learning and memory is shown as poor performances on memory task in human heroin users [5] and opioid-treated rodents [6-8]. Acute or chronic exposure to morphine impairs the function of learning and memory [6, 7]. Long-term potentiation (LTP) is the well-established cellular model of learning and memory,

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and chronic morphine treatment significantly inhibits LTP in rat hippocampus [8]. Recently, it has been proposed that common neurobiological mechanisms and same molecular and cellular events might contribute to drug addiction and the associated learning and memory impairment [9].

The hippocampus is a brain region associated with learning and memory [10]. Increasing evidence demonstrates that it also plays an essential role in opioid dependence. For instance, it has been reported that the hippocampus is functionally involved in morphine-induced conditioned place preference [11-13] and withdrawal behaviours [14-16]. Additionally, it also plays a role in cocaine-seeking behaviour [17] as well as in alcohol tolerance [18]. Thus, the hippocampus appears to be an important region associated with both drug dependence and learning and memory. However, the molecular events occurring in the hippocampus following exposure to abused drugs such as opioids are poorly understood.

Attenuation of several enzyme activities in cell intermediary metabolism such as glycolysis and the tricarboxylic acid (TCA) cycle has been observed in the brain of opiateabusing patients and opiate dependent animals [19-21]. It has also been reported that systemic or intrahippocampal injection of glucose or its metabolite pyruvate attenuates acute morphine-induced memory impairment and increment of locomotor activity [6, 22-25]. In addition, glucose has also been shown to suppress acute morphine withdrawal signs in rodents and in guinea-pig ileum [26-28]. These findings suggest that opioid-induced dependence and memory deficits may be partially attributed to the impairment of hippocampal glucose metabolism as a sequel to attenuation of metabolic enzyme activities.

Opiate dependence is associated with long-term adaptive changes in the brain, which may involve alterations of gene and protein expression in certain brain areas [29]. This study, therefore, was undertaken to examine the effect of chronic morphine treatment on hippocampal protein expression using proteomic analysis and determine whether downregulation of expression of hippocampal energy metabolism enzymes was involved in opiate dependence and associated impairment of learning and memory.

## **Materials and Methods**

#### Animal treatment and morphine withdrawal jumping

Kunming strain male mice (18-22 g) were obtained from Experimental Animal Centre, Chinese Academy of Sciences (Shanghai, China). Mice were housed in groups and maintained on a 12 h light/dark cycle at 22-24 °C with food and water available *ad libitum*. Studies were carried out in accordance with the Guide for Care and Use of laboratory Animals as adopted by the National Institutes of Health. Acute morphine dependence was induced by a single subcutaneous injection of morphine (100 mg/kg). Chronic morphine dependence was rendered by subcutaneous injection of morphine twice daily at 8 h intervals for 10 days as described previously [30]. One group of mice was treated concomitantly with morphine and naltrexone (4 mg/kg, i.p.). Naltrexone was injected 30 min before each morphine injection. Control mice were treated similarly, except that normal saline was used as a substitute for morphine and naltrexone. For assessment of dependence, withdrawal jumping was precipitated by subcutaneous injection of naloxone (2 mg/kg) 2 h following a single (acute) or final (chronic) morphine injection. The jumping frequency of each mouse and the number of mice that jumped in each group within 20 min were recorded. The positive jumping response was defined as jumping more than four times in 20 min. For examination of the effect of glucose on acute and chronic dependence, glucose was intraperitoneally (200 to 1 000 mg/kg) or intrahippocampally (8 nmol per side) injected before single or final morphine injection. For proteomic, mRNA and ATP analysis, animals were killed by decapitation 1 h after a single or final morphine injection. The hippocampus was isolated, frozen in liquid nitrogen and then stored at -80 °C until used.

### Intrahippocampal injection of glucose

Mice were anaesthetized with sodium pentobarbital (35 mg/kg, i.p.), and fixed on a stereotaxic apparatus (SR-5, Narishige, Tokyo, Japan) with mouse adapter and lateral bars. A guide cannulae (0.5 mm outer diameter) made of stainless steel tubing was implanted into the bilateral hippocampus according to the following coordinates measured from bregma: AP: -2.8 mm, L: ±2.5 mm and H: 1.8 mm from the skull [31]. At least 7 days were allowed for recovery from the surgery. Correct placement of cannulae was verified after the experiment. Infusion cannulae (0.25 mm diameter) were gently inserted into the guide cannulae. The injector was connected with a polyethylene catheter tubing to a 1-µl Hamilton syringe driven by a microinjection pump at a rate of 0.1 µl/min. Injection of glucose (8 nmol in 0.2 µl aCSF (artificial cerebrospinal fluid) per side) or aCSF (0.2  $\mu$ l per side) were performed in freely moving mice at a perfusion rate of 0.2 µl/min. To allow diffusion of drug, the infusion cannulae were left in place 1 min following the injection [32].

#### Morris water maze test

This test was performed as previously described [7]. Briefly, a circular pool (diameter 110 cm, height 40 cm) was filled to a depth of 20 cm with water. The water was maintained at  $20 \pm 0.5$  °C and made opaque by the addition of 15-ml India ink. Four equally spaced points around the edge of the pool were designed as four starting positions: east (E), south (S), west (W) and north (N). An escape platform (diameter 6.5 cm) was submerged 1 cm below water surface and placed in the centre of the south and west quadrant. The mouse in the pool was trained to find the platform. Each mouse was trained four times each day. The latency to find the escape platform was measured during each trial. Upon finding and climbing the platform, the mice stayed there for 20 s. If the mice failed to find the platform within 60 s, they were placed on the platform by experimenter and a maximum score of 60 s was given. After rest for 20 s on the platform, the next trial was initiated. For assessment of the effect of glucose or 3-O-methyl-glucose on acute morphine-induced impairment of learning and memory, the task was performed consecutively for 5 days. From day 1 to day 4, each mouse received two injections of saline daily before the training. On the test day, glucose (500 mg/kg, i.p.) or 3-O-methyl-glucose (500 mg/kg, i.p.) was injected

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2 h before morphine injection. Escape latency of each mouse was recorded 20 min after morphine injection (10 mg/kg, s.c.). For test of the effect of glucose on chronic morphine-induced cognitive deficits, mice were chronically treated with morphine for 10 days as described above, and were trained 20 min after second injection of morphine or saline every day. Glucose (500 mg/kg, i.p.) was given 2 h before the last morphine injection. Escape latency of each mouse was recorded 20 min after a single (acute) or final (chronic) morphine injection. To determine the effect of morphine on mice swimming ability and visual discrimination, the swimming time for mice to climb onto the platform was also recorded when the platform was set in a pool filled with clear water to the depth of 20 cm and made visible by mounting a blue-coloured picture on it.

## Sample preparation and two-dimensional gel electrophoresis

Frozen samples of the hippocampus were homogenized in 400 µl lysis solution containing 8 M urea, 4% CHAPS (Bio-Rad, Hercules, CA), 100 mM DTT and 2% Ampholyte 3-10 (Bio-Rad, Hercules, CA), and then centrifuged at 20 000  $\times$  g for 2 h at 4 °C. The supernatants were stored in aliquots at -80 °C until analysed and the protein concentration in supernatant was determined by the Bradford method. Two-dimensional gel electrophoresis (2-DE) was performed as previously reported [33]. Samples of 150 µg protein were applied on 17 cm immobilized pH 4-7 linear gradient strips (Bio-Rad, Hercules, CA). Isoelectric focusing starts at 100 V for 30 min and gradually increased to 5 000 V and kept constant for 12 h. After IEF, the strips were equilibrated twice for 15 min with gently shaking in a solution of Tris-HCl buffer (50 mM, pH 6.8), urea (6 M), glycerol (30%, w/v), SDS (2%, w/v) and DTT (2% w/v). DTT was replaced with 2.5% w/v iodoacetamide (Fluka, Buchs, SG) in the second equilibration. After equilibration, the IPG strips were placed on top of the SDS-polyacrylamide gels (12% T; 0.32% C) (Sigma St. Louis, MO). The gels  $(180 \times 200 \times 1.5 \text{ mm})$  were run at 40 mA per gel, in the vertical Protean II Xi cell (Bio-rad, USA) at 10 °C. Then the gels were silver stained according to the method [34]. Molecular masses were determined by running standard protein markers, covering the range of 14-66 kDa. The pI values were used as given by the supplier of the IPG strips. The silver-stained gels were scanned using a GS-800 Calibrated Imaging Densitometer (Bio-Rad, Hercules, CA) in transmission mode. The protein spots were detected, quantified and matched using the PD-Quest 2D-gel analysis software, Version 7.1 (Bio-Rad, Hercules, CA). The protein spots from different gels were matched and their volumes were determined. The integrated intensities of all spots appearing on the control and morphine master images were compared. Only the spots of which the integrated intensity differed by at least two-fold were statistically analysed and proteins with levels altered significantly (p < 0.05) in the morphine-treated mice as compared to control were reported. Altered proteins were cut out for identification.

#### In-gel digestion and mass spectrometry

Silver-stained protein bands were excised, destained by  $1\% H_2O_2$ in 25 mM ammonium bicarbonate, pH 8.5, followed by reduction and alkylation as previously described [35]. In-gel trypsin digestion (300 ng) was allowed at 37 °C for 18 h. Tryptic peptides were subsequently extracted using three cycles of 5% formic acid/50% acetonitrile at 37 °C for 1 h each. LC-MS/MS analysis was performed using a Micromass Q-TOF Ultima Global (Micromass, Manchester, UK) in electrospray mode interfaced with an Agilent HP1100 CapLC (Agilent Technologies, Palo Alto, CA) before the mass spectrometer. Five millilitres of each digest was loaded onto a Vydac C18 MS column ( $100 \times 0.15$  mm; Grace Vydec, Hesperia, CA) and chromatographic separation was performed at 1 il/min using the following gradient: 0-10% B over 5 min; gradient from 10 to 40% B over 60 min; from 40% to 95% B over 5 min; 95% B held over 5 min (solvent A: 0.2% formic acid in water; solvent B: 0.2% formic acid in acetonitrile). Data-dependent analysis (DDA) method collected tandem MS/MS data for the three most abundant peptide ions observed in the preceding survey scan (*m*/z 300-1990) above a threshold of 10 counts/s. Data were processed using the MassLynx software package (version 4.0) to generate peak list files before in-house licensed Mascot search [36] at http://biospec.nih.gov (MatrixScience Ltd, London, UK).

#### Real-time quantitative polymerase chain reaction

RNA was extracted from the hippocampus harvested from six chronic morphine-treated mice and from six saline-treated mice using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Oligo (dT)-primed first-strand cDNA synthesis was performed with kit for RT-PCR (M-MLV) (Promega, Madison, WI) using 2 µg of total RNA as a template in a total volume of 25 µl. Reaction conditions were set up as described by the manufacturer's manual. The thermal cycling conditions were 94 °C for 2 min, followed by 35 cycles, each consisting of 94 °C for 30 s for melting, 60 °C for 30 s for annealing, and 72 °C for 30 s extension. Experimental samples, standards and no-template controls were all run in triplicate. For quantification of RNA targets, QuantiTect SYBR Green RT-PCR was used, containing SYBR Green I as a fluorescent dye. Specificity of RT-PCR products was verified by checking the product melting curves (every time). To further ascertain the specificity and size of PCR products, the products were run on 2% agarose gel with molecular weight marks. The gel was stained with ethidium bromide.

The primers were designed from the sequences in the GenBank database using Primer Express software (Perkin-Elmer, Boston, MA). The followings are the sequences of primers:

LDH2: forward 5'-TCTGCTCGATTCCGCTAC-3', reverse 5'-CACCTCCTTCCAGTTCTCAC-3';

Fe-S protein 1 of NADH dehydrogenase: forward 5'-TCATTCT-GCGAGGTCTTA-3', reverse 5'-ATAGCCAAGGTCCAAAGC-3';

PDHC-E2: forward 5'-TCCAAAGCGAGAGAGGGGTAA-3', reverse 5'-AGCACCGATTGCCAGAATAC-3';

β-actin (as a housekeeping gene): forward 5'-TTCTTTGC-AGCTCCTTCG-3', reverse 5'-TTCTGACCCATTCCCACC -3'.

All the primers were synthesized by Boya (Boya, Shanghai).

#### ATP determination

ATP levels of the hippocampus derived from different treatment mice were determined using the Bioluminescence detection kit (Promega, USA), according to the manufacturer's instruction. The samples of the hippocampus were weighted, and then grinded in 0.4 M perchlorid acid (10  $\mu$ l/mg wet weight) and centrifuged (10 000 rpm for 10 min). Supernatants were collected and neutralized to pH 7.8 with 2 M K<sub>2</sub>CO<sub>3</sub> and centrifuged (10 000 rpm. for 10 min. Samples were then 1 000-fold diluted in distilled water and 180  $\mu$ l aliquot was added to wells of a 96-well reaction plate. This plate was loaded into the luminometer (Polar star galaxy) and 20  $\mu$ l of the rL/L Reagent were added to each well by means of an injection pump, and the samples were measured. Immediately after addition of the rL/L Reagent, ATP was monitored over a period of 10 s. ATP concentration was calculated according to an ATP standard curve (from 10<sup>-11</sup> to 10<sup>-7</sup> M). Data are expressed using the relative concentrations of ATP.

## Statistical analysis

Data are presented as means  $\pm$  SEM of at least three independent experiments. Statistical difference between treatment groups was analysed by one-way ANOVA followed by Newman-Keuls test or Student's *t*-test.

## Results

# Decreased expression of Fe-S protein 1 of NADH dehydrogenase, PDHC-E2 and LDH2 in chronic morphine-treated mouse hippocampus

The hippocampal extracts from control (saline treated), acute and chronic morphine-treated mice were separated by 2-DE followed by silver staining. Proteins displaying altered levels were quantified from the 2-DE gels using PD-Quest software and identified by Q-TOF mass spectrometer. 2-DE separation and silver staining revealed that there were 1240 spots in the gel of hippocampal sample of salinetreated mice (Figure 1, upper panel to Figure 1A). A similar pattern of protein expression was detected in the hippocampal sample of chronic morphine-treated mice (Figure 1, lower panel to Figure 1B). Most spots were distributed at positions between pH 4 and 7, and with molecular weights between 20 and 100 kDa. Comparison of the intensity of protein spots between chronic morphine- and saline-treated mice indicated that seven proteins were decreased and one was increased significantly (p < 0.05) in the hippocampus from chronic morphine-treated mice (Table 1).

Interestingly, among the seven proteins that were significantly decreased, three were identified as energy metabolism enzymes, namely Fe-S protein 1 of NADH dehydrogenase, dihydrolipoamide acetyltransferase or E2 component of the pyruvate dehydrogenase complex (PDHC-E2) and lactate dehydrogenase 2 (LDH2) (Figure 2; Table 2). Similar to the hippocampus from saline-treated mice, changes in the expression of these proteins were not observed in the hippocampus from mice that were subjected to a single acute injection with a dose of 100 mg/kg of morphine (data not shown). To address whether the downregulation of the expression of these three proteins (Fe-S protein 1 of NADH dehydrogenase, PDHC-E2 and LDH2) was mediated by opioid receptors, the effect of opioid receptor antagonist naltrexone (4 mg/kg, i.p.) was examined. As expected, administration of naltrexone before each morphine injection prevented morphine-mediated decrease in the expression of these three proteins (Figure 3).

# Reduced levels of mRNAs of Fe-S protein 1 of NADH dehydrogenase, PDHC-E2 and LDH2 in chronic morphinetreated mouse hippocampus

To further confirm the proteomic analysis results and to examine the underlying mechanisms of reduced expression of these three proteins, we determined whether the levels of the corresponding mRNAs of these proteins were affected by chronic morphine treatment, using quanitative real time RT-PCR. Figure 4A shows the detection of specific RT-PCR products on an agarose gel, and the specificity was further validated by constructing a melting temperature curve (data not shown). Our results showed that the transcripts for Fe-S protein 1 of NADH dehydrogenase, PDHC-E2 and LDH2 were significantly decreased (by 1.86-, 1.67- and 1.56-fold respectively) in the hippocampus of chronic morphinetreated mice as compared to saline-treated control. Taken together, the results of our protein and mRNA analyses support the notion that chronic morphine treatment leads to reduced expression of these three metabolic enzymes (Fe-S protein 1 of NADH dehydrogenase, PDHC-E2 and LDH2).

# Reduction of ATP production and impairment of glucose metabolism in the hippocampus of chronic but not acute morphine-treated mice

Fe-S protein 1 of NADH dehvdrogenase, PDHC-E2 and LDH2 are associated with energy metabolism and ATP production. To further substantiate the results from the proteomic analysis, we examined glucose metabolism and ATP levels in the hippocampus from control, acute and chronic morphine-treated mice. As shown in Figure 5A, the levels of ATP in the hippocampus from chronic morphine-treated mice were significantly lower than that in the saline-treated control, suggesting that energy metabolism was impaired in the hippocampus of chronic morphine-treated mice. The reduction of ATP production by chronic morphine treatment could be prevented by concomitant administration of naltrexone. In contrast, significant reduction of ATP levels was not observed in the hippocampus from acute morphine-treated mice (Figure 5A, left panel). Intraperitoneal injection of D-glucose (200 mg/kg) significantly increased ATP levels in the hippocampus from acute morphine-treated mice but not from chronic morphine-treated mice (Figure 5B), further suggesting the impairment of glucose metabolism in the hippocampus of chronic morphine-treated mice. Intraperitoneal injection of the same dose of 3-O-methyl-glucose, a non-metabolizable sugar, failed to increase ATP levels in the hippocampus from acute morphine-treated mice (Figure 5B), suggesting that glucose metabolism is necessary for the observed increase of ATP levels. Taken together, these results suggest that downregulation of Fe-S protein 1 of



**Figure 1** Representative 2-DE gel images of the hippocampal proteins from mice differentially treated with saline (**A**) or morphine (**B**). Mice were treated with saline or an increasing dose (from 20 to 100 mg/kg, s.c.) of morphine for 10 days. Hippocampal protein (150 µg) was separated on PH 4-7 linear IPG strips (170 cm) followed by separation on 12% SDS-PADE gels as described in Materials and Methods. The gels were visualized using silver staining. The samples for 2-DE were prepared from hippocampus of nine mice. The horizontal dimension represents a PH gradient of 4-7, and the vertical dimension is indicated by molecular markers. Labelled spots (**A-C**) are proteins that were differentially expressed in the hippocampus from mice differentially treated with saline or morphine.

Table 1 Eight differentially expressed proteins between chronic saline- and morphine-treated mice

Spot no.	Observed pI/	Abunda	ance	Folds of changes	P-value
	Mr (kDa)	Control	Morphine		
Ι	5.5/79	0.054±0.015	$0.018 \pm 0.018$	-3.00	0.009
II	5.9/73	0.170±0.047	$0.048 \pm 0.025$	-3.54	0.001
III	5.7/59	0.437±0.075	0.170±0.059	-2.57	0.001
IV	5.7/36	0.277±0.177	$0.083 \pm 0.073$	-3.37	0.035
V	4.5/58.6	0.063±0.012	0.030±0.012	-2.11	0.019
VI	4.7/49.7	0.040±0.010	$0.010 \pm 0.000$	-4.00	0.007
VII	5.9/38.4	0.127±0.012	0.037±0.021	-3.45	0.003
VIII	5.6/30.0	0.043±0.021	0.101±0.025	+2.52	0.007

The silver-stained gels were scanned using a GS-800 Calibrated Imaging Densitometer in transmission mode. The protein spots were matched using the PD-Quest 2D-gel analysis software, and only the spots of which the integrated intensity differed by at least two-folds were statistically analysed. The integrated intensities of these spots appearing on the control and morphine images were compared.  $201 \times 121$  mm ( $72 \times 72$  DPI).

complex I, PDHC-E2 and LDH2 in the hippocampus of chronic morphine-treated mice leads to a decrease of ATP production due to impaired glucose metabolism.

# Intrahippocampal injection of D-glucose suppressed naloxone-precipitated morphine withdrawal jumping in acute but not chronic morphine-treated mice

To determine the role of impaired hippocampal glucose metabolism in morphine dependence, we next examined the effect of intrahippocampal glucose administration on naloxone-precipitated morphine withdrawal jumping in both acute and chronic morphine-treated mice. Among withdrawal behaviours in opiate dependence rodents (especially, mice), jumping is widely considered as the most sensitive and reliable index and is commonly used to test opiate dependence state [2]. In agreement with previous studies [2-4], both acute and chronic treatment of mice with morphine induced a robust withdrawal jumping following naloxone precipitation, and this effect could be significantly suppressed by concomitant administration of naltrexone with morphine (Figure 6).

Next, the effects of glucose administration on acute and chronic morphine-induced withdrawal jumping were determined. Intrahippocampal injection of D-glucose (8 nmol per side) before morphine administration significantly reduced the numbers of jumping mice and jumping frequency of



**Figure 2** Decreased expression of Fe-S protein 1 of NADH dehydrogenase, PDHC-E2 and LDH2 in the hippocampus from mice chronically treated with morphine. (A) 2-DE images of the hippocampal proteins derived from the saline-, and chronic morphine-treated mice. (B) Quantification of the alterations of protein expression between the two groups. Mice were treated with saline or an increasing dose (from 20 to 100 mg/kg, s.c.) of morphine for 10 days. The hippocampus extracts from mice differentially treated with saline or morphine were separated by 2-DE followed by silver staining. Protein levels were quantified using PD-Quest software. Data are presented as means  $\pm$  SEM of at least three separate experiments (n = 9). \*\*p < 0.01 versus saline-treated mice.

Table 2 Identification of the proteins differentially expressed between saline- and morphine-treated mouse hippocampus by mass spectrom
etry

Spot no.	Proteins identified	Accession	Theoretical pI	Mascot total	Identified peptides
		no. NCBI	/Mr (kDa)	MOWSE	
Ι	Fe-S protein 1	21704020	5.5/79	75	SATYVNTEGRFASEIAGV
					DDLGTTGR
					ALSEIAGITLPYDTLDQVR
III	PDHC-E2	16580128	5.7/59	167	ILVPEGTR
					VFVSPLAK
					DIDSFVPSK
					GLETIASDVVSLASK
IV	LDH2	6678674	5.7/36	168	LNLVQR
					IVVVTAGVR
					GLTSVINQK
					VIGSGC*NLDSAR

Fe-S protein 1 = Fe-S protein 1 of NADH dehydrogenase; PDHC-E2 = E2 component of the pyruvate dehydrogenase complex; LDH2 = lactate dehydrogenase 2. Silver-stained protein bands were excised, trypsinized, extracted and analysed with Q-TOF mass spectrometer as described in Materials and Methods. Data were processed using the MassLynx software package to generate peak list files before in-house licensed Mascot search.

each mouse in acute morphine-treated groups (Figure 6A). However, intrahippocampal injection of D-glucose failed to inhibit naloxone-precipitated morphine withdrawal jumping in chronic morphine-treated mice (Figure 6B). We also examined the effect of the non-metabolizable sugar 3-O-methyl-glucose, and found that intrahippocampal injection of the same dose of 3-O-methyl-glucose was unable to inhibit morphine withdrawal jumping in acute morphine-treated mice (Figure 6A). These results suggest that normal energy metabolism is a premise for glucose to suppress morphine withdrawal jumping. In addition, intraperitoneal injection of D-glucose (200, 500 and 1 000 mg/kg) before morphine administration also significantly inhibited morphine withdrawal jumping in acute morphinetreated mice (data not shown). It is likely that certain metabolites of glucose may exert an inhibitory effect on the



**Figure 3** Naltrexone reversed chronic morphine-induced downregulation of Fe-S protein 1 of NADH dehydrogenase, PDHC-E2 and LDH2. (A) 2-DE images of the hippocampal proteins from chronic morphine-, and chronic morphine plus naltrexone-treated mice. (B) Quantification of the alterations of protein expression between the two groups. Mice were treated with an increasing dose (from 20 to 100 mg/kg, s.c.) of morphine alone or morphine in combination with naltrexone (4 mg/kg, i.p.) for 10 days. The hippocampus extracts from differentially treated mice were separated by 2-DE followed by silver staining. Proteins displaying altered levels were quantified using PD-Quest software. Data are presented as means  $\pm$  SEM of at least three independent experiments (n = 9). \*\*p < 0.01 versus combination-treated mice.



**Figure 4** Downregulation of mRNAs corresponding to Fe-S protein 1 of NADH dehydrogenase, PDHC-E2 and LDH2 in the hippocampus of chronic morphine-treated mice. (A) Gel electrophoresis of PCR products from mouse hippocampal cDNA. A single band of the expected size for each gene was detected on agarose gel. G1, G2, G3 and G4 represent the RT-PCR products corresponding to mRNAs of LDH2 (192 bp), Fe-S protein 1 of NADH dehydrogenase (210 bp), PDHC-E2 (141 bp) and  $\beta$ -actin (197 bp), respectively. The marker lane shows a ladder of 14 bands from 100 to 3 000 bp (lane1). (B) Fold of reduction of mRNAs of Fe-S protein 1 of NADH dehydrogenase, PDHC-E2 and LDH2 in the hippocampus from chronic morphine-treated mice. Data are presented as means  $\pm$  SEM of three independent experiments (n = 6). \*\*p < 0.01 versus saline-treated mice.

development and/or expression morphine dependence and that the impairment of hippocampal glucose metabolism would thus contribute to morphine dependence.

## Intraperitoneal injection of D-glucose suppressed memory impairment in acute but not in chronic morphine-treated mice

Opioid dependence and learning and memory impairment have been proposed to share common molecular and cellular bases [9]. We thus examined the effect of glucose injection on memory impairment induced by acute or chronic morphine treatment. Since systemic and central injection of D-glucose produces similar behaviour effects [37] and peripheral glucose injection can increase ATP levels in the hippocampus (Figure 5), we administered D-glucose to mice by intraperitoneal injection. In agreement with previous studies [7, 8], acute and chronic exposure of mice to morphine impaired their performance in Morris water maze



Figure 5 Reduction of ATP levels and impairment of glucose metabolism in the hippocampus derived from mice chronically treated with morphine. (A), Decrease of the hippocampal ATP levels by chronic but not acute morphine treatment and reversal of the chronic morphine effect by concomitant administration of naltrexone. (B), Increase of the hippocampal ATP levels by intraperitoneal injection of D-glucose (200 mg/kg, i.p.) in acute morphine-treated but not in chronic morphine-treated mice. Mice were treated either acutely with a single dose of morphine (100 mg/kg, s.c.) or chronically with an increasing dose of morphine (s.c.) alone or in combination with naltrexone (4 mg/kg, i.p.) for 10 days. ATP levels in the hippocampus from differentially treated mice were measured as described in Materials and Methods. Data are presented as means  $\pm$  SEM of at least three independent experiments (n = 6). \*p < 0.05 versus saline-treated mice (A) or versus acute morphine-treated mice without glucose injection (B). Sal: saline; Mor: morphine; Glu: D-glucose; mGlu: 3-O-methyl-glucose; Nal: naltrexone.

task. As shown in Figure 7, acute exposure of mice to morphine at the single dose of 10 mg/kg significantly prolonged the escape latency. Injection of D-glucose (500 mg/kg, i.p.) but not 3-O-methyl-glucose before morphine administration markedly shortened the escape latency. Consecutive exposure of mice to an escalating dose of morphine for 10 days also significantly prolonged their escape latency. However, in contrast to the effect of glucose on acute morphine-induced memory deficits, injection of D-glucose (500 mg/kg, i.p.) before final morphine injection failed to shorten the escape latency in chronic morphine-treated mice (Figure 7). The failure to alleviate morphine-induced memory impairment by glucose in chronic morphine-treated mice

and by 3-O-methyl-glucose in acute morphine-treated mice suggests that glucose metabolism appears to be necessary for its antagonism of morphine-induced memory deficits. The observed latency difference between morphine-treated and control mice could not be explained by the effect of morphine on motor because there was no significant difference between saline- and morphine-treated mice in their swimming ability and visual discrimination for performing the task (data not shown).

## Discussion

With proteomic analysis we found that chronic exposure to morphine resulted in a significant downregulation of the levels of Fe-S protein 1 of NADH dehydrogenase, PDHC-E2 and LDH2 in the hippocampus. The finding was further confirmed by reduced expression of the corresponding mRNAs for these proteins and by pronounced reduction of ATP levels in the hippocampus of chronic morphine-treated mice. More importantly, our findings provide the first evidence showing that impairment of glucose metabolism in the hippocampus, as a result of reduced expression of these three proteins, was involved in the development of morphine withdrawal jumping and memory impairment.

Fe-S protein 1 of NADH dehydrogenase, PDHC-E2 and LDH2 are important enzymes or coenzyme for energy metabolism. Fe-S protein 1 is the coenzyme of NADH dehvdrogenase, playing a key role in the transport of electrons from NADH to ubiquinone in the respiratory chain for ATP production. As one of the three enzymes of the pyruvate dehydrogenase complex, PDHC-E2 is necessary for the conversion of pyruvate to acetyl-CoA for the TCA cycle and serves as the bridge linking glycolysis and the TCA cycle in glucose metabolism. LDH2, a key enzyme of glycolysis, catalyses the reduction of pyruvate by NADH to form lactate, enabling the regeneration of NAD<sup>+</sup> to help fulfil the energy demand from glycolysis. Hence, downregulation of these proteins would result in impairment of energy metabolism and decrease of ATP production. This is supported by the fact that significantly lower ATP levels and an impaired ability to covert glucose to ATP were observed in the hippocampus of chronic morphine-treated mice. Our findings also offer a possible explanation for previous reports showing reduction of metabolic enzyme activities and increase of serum glucose levels in human and rodents upon chronic exposure to opiates [19, 20, 21, 38].

Both acute and chronic exposures to morphine lead to dependence [2-4]. Morphine dependence is manifested by characteristic withdrawal syndrome of multiple aversive behavioural signs. Among these withdrawal behaviours, jumping is widely considered as the most sensitive and reliable index and is commonly used to test opiate de-



**Figure 6** Effect of D-glucose on naloxone-precipitated morphine withdrawal in acute and chronic morphine-treated mice. (A) Inhibition of naloxone-precipitated morphine withdrawal jumping by administration of D-glucose but not by 3-*O*-methyl-glucose in acute morphine-treated mice. (B) Failure of suppression of naloxone-precipitated withdrawal jumping by D-glucose in chronic morphine-treated mice. (B) Failure of suppression of naloxone-precipitated withdrawal jumping by D-glucose in chronic morphine-treated mice. (B) Failure of suppression of naloxone-precipitated withdrawal jumping by D-glucose in chronic morphine-treated mice. (B) Failure of suppression of naloxone-precipitated withdrawal jumping by D-glucose in chronic morphine-treated mice. (Sc.) alone or in combination with naltrexone (4 mg/kg, i.p.) for 10 days. D-glucose or 3-*O*-methyl-glucose (8 nmol per side) was injected intrahippocampally before morphine (acute) or final morphine administration (chronic), followed by precipitation with naloxone (2 mg/kg, i.p.). Withdrawal jumping was detected as described in Materials and Methods. Data are presented as means  $\pm$  SEM of at least three independent experiments (n = 10). \*p < 0.05, \*\*p < 0.01 versus acute morphine-treated mice (A) or versus chronic morphine-treated mice (B). Mor: morphine; Nal: naltrexone; Glu: glucose; mGlu: 3-*O*-methyl-glucose.



**Figure 7** Improvement of acute but not chronic morphine treatment-induced memory deficits by D-glucose. Mice were treated either acutely with a single dose of morphine (10 mg/kg, s.c.) or chronically with morphine for 10 days. Escape latency of each mouse was recorded 20 min after morphine administration with the Morris water maze test as described in Materials and Methods. D-glucose or 3-*O*-methyl-glucose (500 mg/kg) was given by intraperitoneal injection before morphine (acute group) or final morphine administration (chronic group). Data are presented as means  $\pm$  SEM (n =12). \*\*p < 0.01 versus the saline group, ##p < 0.01 versus the acute morphine group, \*p < 0.05 versus the saline group. Mor: morphine; Nal: naltrexone; Glu: glucose; mGlu: 3-*O*-methyl-glucose.

pendence state of the rodents (especially in mice) [2]. Our present study demonstrated that intrahippocampal or intraperitoneal injection of glucose before morphine administration significantly suppressed naloxone-precipitated morphine withdrawal jumping in acute morphine-treated, but not in chronic morphine-treated mice, suggesting that glucose metabolism is necessary for its antagonism of morphine withdrawal jumping and this antagonism may be mediated by its metabolites. Indeed, ATP and its metabolites such as adenosine have been reported to suppress naloxone-precipitated morphine withdrawal symptoms [39, 40]. The notion is also supported by the observation that intraperitoneal or intrahippocampal injection of the non-metabolizable sugar, 3-O-methyl-glucose, failed to suppress naloxone-precipitated withdrawal jumping in acute single morphine-treated mice. The different effects of glucose on morphine withdrawal jumping in acute and chronic morphine-treated mice may be attributed to the distinct downregulation of the three energy metabolism related proteins in chronic morphine-treated mice. Due to the downregulation of these proteins in the hippocampus of chronic morphine-treated mice, conversion of glucose to ATP and other metabolites would be impaired, and as a result it fails to inhibit morphine withdrawal jumping in these animals. Our results suggest that downregulation of the three proteins associated with energy metabolism in the hippocampus is involved in morphine dependence. This notion was further strengthened by the observation that preventing the downregulation of these three proteins by concomitant use of naltrexone during chronic morphine treatment also prevented morphine withdrawal jumping in these mice.

The Morris water maze task has been established as a useful behavioural test for spatial learning associated with the hippocampal function [41]. Consistent with previous studies [7, 42], our results showed that glucose suppressed acute morphine-induced impairment of learning and memory, whereas it failed to alleviate learning and memory impairment induced by chronic morphine treatment. The suppressive effect of glucose on acute morphine-induced withdrawal jumping and impairment of learning and memory suggests that common molecular and cellular events might be involved in the development or expression of these symptoms following exposure to morphine, and that alteration of glucose metabolism may be involved in the initiation of these molecular and cellular events.

One of the possible explanations for glucose suppression of morphine withdrawal jumping and memory impairment in acute morphine-treated mice is that certain metabolites or intermediate metabolites of glucose may exert an inhibitory effect on morphine-induced dependence and memory impairment. These metabolites or intermediate metabolites might either act as substrates, augmenting the synthesis of certain neurotransmitters such as acetylcholine, or act as neurotransmitters or neuromodulators, modulating the release of certain neurotransmitters (e.g., glutamate, GABA, dopamine, acetylcholine and noradrenaline). It is well established that acetylcholine is crucially involved in modulation of learning and memory [10]. It has also been shown that the attenuation of central cholinergic activity is involved in the expression of opioid withdrawal symptom [43, 44] and impairment of learning and memory [7]. Morphine has been shown to inhibit hippocampal acetylcholine release [45, 46]. Glucose, administrated peripherally or intrahippocampally, could enhance hippocampal acetylcholine release [47, 48] by promoting the synthesis of acetylcholine through increasing the levels of acetyl-CoA, a precursor of acetylcholine [37].

Neurotransmitters such as glutamate and GABA play a crucial role in the development and/or expression of opioid dependence [49, 50]. In the hippocampus, ATP and its metabolite adenosine have been shown to play an important role in modulation of glutamate and GABA release [51-53]. Chronic exposure to opioids such as morphine might result in increment of glutamate release and decrement of GABA release through alteration of ATP and adenosine production in the hippocampus, as a result of impairment of glucose metabolism, thereby leading to the development of opioid dependence. In support of this notion, substantial evidence shows that NMDA receptor antagonists and GABA<sub>B</sub> receptor agonists prevent the development of opioid dependence and suppress opioid withdrawal signs [49, 50]. Meanwhile, accumulating evidence demonstrates that increase of extracellular adenosine levels by adenosine kinase inhibitor and activation of both A1 and A2A receptors by their selective agonists attenuate the incidence of some of the morphine withdrawal signs, such as jumping, wetdog shakes and diarrhoea, whereas antagonists exacerbate the symptoms [40, 54].

In summary, the results of this study suggest that reduced expression of three energy metabolism enzymes and impaired glucose metabolism in the hippocampus of chronic morphine-treated mice were involved in the development and/or expression of the behavioural abnormalities associated with dependence and memory deficits. The effect of glucose on morphine withdrawal jumping is similar to that on memory impairment in acute morphine-treated mice, supporting that morphine-induced memory impairment and dependence may share common molecular and cellular mechanisms. Changes in certain glucose metabolites might be involved in the initiation of these molecular and cellular events. Our results here reveal a new aspect of the mechanisms of opiate dependence. Further studies are necessary to ascertain the metabolites and to elucidate the mechanisms underlying their effects.

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