南台科技大學

生物科技系碩士在職專班
碩士學位論文

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Differential expression of nucleus accumbens proteome in a porcine model of morphine tolerance

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本論文業經審查及口試合格特此證明

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摘　　要

嗎啡可有效處理中度至嚴重疼痛，但長期使用可導致對止痛效果的耐受性，此現象源於尚未完全被瞭解的神經適應及生化上的複雜變化。伏隔核(nucleus accumbens, NAc)是腦中已知與嗎啡耐受性的發生有關的區域之一。在本研究中，我們以蘭嶼迷你猪(Lanyu miniature pigs, *Sus scrofa domesticus*)為實驗動物，研究 NAc 的蛋白質體與嗎啡耐受性之間的關係。嗎啡組與對照組各 6 隻動物。嗎啡組以每天在腦脊髓膜內注射兩次鹽酸嗎啡 75 μg/kg 共 10 天來誘發對止痛效果的耐受性；對照組則以生理食鹽水取代嗎啡。嗎啡耐受性的發生與否是以測量對熱刺激的反應時間長短來評估。以二維差異膠體電泳(two-dimensional difference in gel electrophoresis, 2D-DIGE)比較兩組之間的 NAc 蛋白質含量。針對約 750 個蛋白質點進行統計分析，發現其中 22 個蛋白質點的含量呈現顯著差異($P < 0.05$)。然後以液相層析串聯質譜儀(LC-MS/MS)加上資料庫比對來鑑定上述的失調蛋白質。在嗎啡組中表現量增加的蛋白質包括 soluble epoxide hydrolase 及 eukaryotic elongation factor 1 γ-like protein 等；在嗎啡組中表現量減少的則有 calcineurin catalytic subunit δ isoform、heat shock 60kDa protein、α-internexin、creatine kinase B-type 等。這些發現可能有助於釐清與鴉片類藥物耐受性有關的分子機制之間的複雜互動。

關鍵字：嗎啡、藥物耐受性、伏隔核、家豬、蛋白質體學、二維差異膠體電泳。

ABSTRACT

Morphine is effective in managing moderate to severe pain, but its chronic use can lead to analgesic tolerance, a phenomenon of complex underlying neuroadaptive and biochemical changes not yet fully elucidated. The nucleus accumbens (NAc) is one of the brain regions known to be involved in the development of morphine tolerance. In the present study we aimed to examine the NAc proteome as a function of morphine tolerance in Lanyu miniature pigs (*Sus scrofa domesticus*). The morphine group and the control group each consisted of 6 animals. Analgesic tolerance in the morphine group was induced by intrathecal injection of morphine hydrochloride 75 μg/kg twice daily for 10 days, while saline was injected in the control group. The development of morphine tolerance was assessed by measuring the response time to a thermal stimulation. Two-dimensional difference in gel electrophoresis (2D-DIGE) was used to compare the abundance of NAc proteins between the two groups. Among ~750 protein spots subjected to statistical analysis, 22 spots showed a significant change ($P < 0.05$) in abundance. These de-regulated proteins were further identified using liquid chromatography tandem mass spectrometry (LC-MS/MS) followed by database interrogation. Proteins that were up-regulated in the morphine group included soluble epoxide hydrolase and eukaryotic elongation factor 1 γ-like protein, whereas proteins that were down-regulated included calcineurin catalytic subunit δ isoform, heat shock 60kDa protein, α-internexin and creatine kinase B-type. These findings may help clarify the interplay of molecular events involved in opioid tolerance.

Keywords: morphine; drug tolerance; nucleus accumbens; *Sus scrofa domesticus*; proteomics; 2D-DIGE

誌　　謝

兩年半之前在奇美醫學中心王志中副院長的鞭策下報考碩士在職專班，從此我的人生開啟了另一扇窗。感謝王副院長在本研究進行過程中的鼓勵與大力支持。

恩師吳定峰教授在我陷入研究瓶頸時往往一針見血點出問題的核心，讓我豁然開朗，燃起熊熊鬥志繼續堅持下去，屢次體會到「山窮水盡疑無路，柳暗花明又一村」的喜悅。恩師的積極樂觀與愛台灣的心，更是我的表率。

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本論文可說是奇美醫學中心製藥科技研究室與恩師的分子生物暨蛋白質體研究室合作的成果。感謝製藥科技研究室的諸位好伙伴：謝介平醫師、褚錦承醫師、馮炳勳醫師、黃羿喬、葉乃楨、侯佳慧、王詩維；感謝恩師研究室的徐園堤、周書政、黃麗倩、簡嵐翔、黃泓誠、吳建宏等人有形無形的幫忙。也要感謝陽明大學生化暨分子生物研究所蔡有光老師協助完成本論文中的 LC-MS/MS 實驗。本論文的每個字、每張圖，都是大家的心血結晶；若有任何的榮耀，都是屬於大家的，缺點則該歸咎於我個人的思慮不周與努力不夠。

在這兩年內，戴謙校長、系主任陳啟楨教授及二十多位老師在課堂上使盡渾身解數用心教學，讓身為學生的我們不敢鬆懈。而每位在職專班的同學都是在工作與家庭的夾縫中努力為宇宙的真理而奮鬥，在我的眼中，你們都是勇士！也要感謝同學們讓我在過去一年中擔任班代，有幸為大家服務。特別謝謝同學李凱莉自動自發到我的論文口試會場熱心幫忙佈置並傳授經驗。我也要感謝同事們在這兩年內容忍我三番兩次調班。

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如果本論文的成果能對人類有點貢獻，我還要感謝為了本研究而犧牲的蘭嶼豬英靈們：「作豬作鼠無了時，後出世作好人家囝兒。」尚饗！

回顧這兩年半，可謂點滴在心頭。歡笑也罷，淚水也罷，無論是得是失，皆屬奇恩異典。「萬事都互相效力，叫愛神的人得益處。」跌跌撞撞之後終於鬆了一口氣，除了感恩，還是感恩。

王立楷　謹誌於台灣台南

2009 年 8 月 7 日

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ABBREVIATIONS

2D-DIGE	two-dimensional difference in gel electrophoresis
2-DE	two-dimensional electrophoresis
7-OH-DPAT	7-hydroxydipropylaminotetralin
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate
AMPPNP	5'-adenylylimido-diphosphate
AOP-1	antioxidant protein isoform 1
AUC	area under the curve
BVA	biological variation analysis
cAMP	cyclic adenosine 3',5'-monophosphate; cyclic AMP
CaN	calcineurin
CaN A	calcineurin catalytic subunit
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate
CK-B	creatine kinase B-type
CNS	central nervous system
CREB	cAMP response element-binding protein
CTR	cutaneous trunci reflex
D3R	dopamine D3 receptor
DARPP-32	dopamine- and cAMP-regulated phosphoprotein, 32 kDa
DIA	differential in-gel analysis
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
eEF1γ	eukaryotic elongation factor 1 γ
EET	epoxyeicosatrienoic acid
EF-Tu	elongation factor tu
GFAP	glial fibrillary acidic protein
HPLC	high performance liquid chromatography

hsp60	heat shock 60kDa protein
IEF	isoelectric focusing
IF	intermediate filament
IPG	immobilized pH gradient
KIF	kinesin superfamily protein
LC-MS/MS	liquid chromatography tandem mass spectrometry
mAb	monoclonal antibody
MAPK	mitogen-activated protein kinase
MDS	mesolimbic dopamine system
Mr	molecular weight
MS	mass spectrometry
MW	molecular weight
NAc	nucleus accumbens
NCBI	National Center for Biotechnology Information
NMDA	N-methyl-D-aspartate
NR	non-redundant
pI	isoelectric point
PMSF	phenylmethylsulphonyl fluoride
PP1	protein phosphatase 1
PPP3	protein phosphatase 3
PRDX	peroxiredoxin
PVDF	polyvinylidene fluoride
SBP1	selenium-binding protein 1
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEH	soluble epoxide hydrolase
SVP	synaptic vesicle precursor
TAPS	Taitung Animal Propagation Station
Tris	tris(hydroxymethyl)aminomethane
VTA	ventral tegmental area

第一章　緒論

嗎啡具有良好的止痛效果，臨床上被廣泛用於處理各種中度及嚴重疼痛，但長期使用可導致對止痛效果的耐受性(analgesic tolerance)，此現象源於中樞神經系統中尚未完全被瞭解的神經適應及生化上的複雜變化。

目前咸信伏隔核(nucleus accumbens, NAc)是腦中與嗎啡及其他藥物的成癮性有關的區域之一。諸多研究結果支持此說法，例如：嗎啡被報告過會改變伏隔核神經元的顯微構造、減少伏隔核神經元表面所表現的α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA)接受器及增加伏隔核的細胞外多巴胺(dopamine)濃度。

對於嗎啡耐受性的大多數動物研究皆以齧齒類動物為實驗動物，除了費用上的考量，也便於以不同個體重複實驗。從另一方面來說，長久以來豬被認為是生醫研究的理想實驗動物，其優點包括：一、豬在解剖、生理、代謝各方面比齧齒類動物更接近人類；二、豬的腦部較大，足以容易地定位及取得特定腦核；三、與靈長類動物相比，豬的取得及豢養費用沒有那麼可觀。已有研究顯示嗎啡可引發家豬對於抗傷害感受(antinociception)的耐受性。

對嗎啡類藥物的耐受性涉及多種訊號傳遞成分之間的動態交互作用，極為複雜；而且耐受性的發生機轉可能決定於細胞當時的生理狀態。神經科學的傳統研究方法是一次檢驗一個或數個蛋白質；蛋白質體學(proteomics)的研究方法可以全面探索與對嗎啡類藥物的耐受性有關的多種蛋白質同時發生的改變，可能得以從中發現全新的蛋白質標記以供進一步研究。

為了找出與嗎啡耐受性有因果關係而呈現差異表現(differential expression)的伏隔核蛋白質，本研究採用脊髓腔內(intrathecal)注射嗎啡引發抗傷害感受耐受性的豬動物模式，以二維差異膠體電泳(two-dimensional difference in gel electrophoresis, 2D-DIGE)及液相層析串聯質譜儀(liquid chromatography tandem mass spectrometry,

LC-MS/MS)來鑑定伏隔核的蛋白質體，並以西方墨點法(Western blotting)確認呈現差異表現的部分蛋白質。

Chapter 1
INTRODUCTION

1.1 Background and purpose

Morphine, one of the most potent analgesics, is used to manage moderate to severe pain from a wide variety of clinical entities. Its prolonged use, however, is complicated with side effects such as analgesic tolerance. Tolerance is considered to be the results of complex adaptive and biochemical changes that take place in the central nervous system (CNS), but the precise mechanisms underlying this neuroplasticity[1] are not fully understood and warrant further investigation. It is believed that the nucleus accumbens (NAc), a part of the mesolimbic dopamine system (MDS), is one of the brain loci associated with the pathogenesis of opioid tolerance.[2,3]

Opioid tolerance is so complex that it involves dynamic interplay between a diversity of signaling components; besides, tolerance mechanisms may be contingent on ongoing cellular physiology.[4] Traditional approaches in neuroscience are hypotheses-based methods that examine only one or several proteins at a time, but proteomics approach, a screening technology and open discovery method, can profile large numbers of global, concurrent protein changes associated with opioid tolerance and may provide novel protein targets for further studies in this field.[5]

To identify differential expression of the NAc proteins causally related to morphine tolerance, we used a pig model of intrathecal morphine-induced antinociceptive tolerance and examined the NAc proteome by two-dimensional difference in gel electrophoresis (2D-DIGE) followed by identification of differentially expressed proteins by liquid chromatography tandem mass spectrometry (LC-MS/MS). Some de-regulated proteins were verified by Western blotting.

1.2 Morphine tolerance and NAc

Morphine analgesic tolerance denotes diminished analgesic efficacy with repeated morphine use so that progressively higher doses are needed to achieve the same analgesic effect.[6-8] This phenomenon potentially limits the clinical usability of morphine. Genetic polymorphism may explain the wide inter-individual variability in the manifestations of morphine tolerance.[9]

Possible molecular mechanisms involved in opioid tolerance include:[9-11]

(a) Receptor tolerance that involves desensitization and subsequent internalization of the opioid receptors;

(b) Cellular tolerance that involves adenylate cyclase–cyclic AMP (cAMP)–protein kinase A–cAMP response element-binding protein (CREB), mitogen-activated protein kinases (MAPK) or protein kinase C cascades;

(c) System tolerance that involves "anti-opioid" systems (such as N-methyl-D-aspartate (NMDA) receptor[1,3] and nitric oxide synthase) and changes in glial function;

(d) Synaptic plasticity represented by processes of long-term potentiation and long-term depression that involve changes in α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptor subunits at synapses.

The reinforcing effects of morphine and other drugs of abuse are believed to be mediated by the MDS, where the dopaminergic neurons in the ventral tegmental area (VTA) project primarily to the NAc (also called the ventral striatum).[8,12-16] There have been numerous studies supporting the NAc as a site of opioid rewarding actions,[14,17] which have been associated with changed neuronal microstructure,[18] decreased surface expression of AMPA receptors[19] and increased extracellular dopamine concentrations,[17] for example, in the NAc. In fact, the NAc is considered a site where the reinforcing effects of most (possibly all) drugs of abuse converge.[2] That is why the NAc was chosen as the target of proteomic analysis in the present study.

1.3 Animal model

Most of the animal studies on morphine tolerance have been done in rodents because of expense considerations and convenience of including more biological replicates. On the other hand, pigs have long been recognized as ideal experimental animals in biomedical research[20,21] and offer several advantages. First, compared with rodents, they are more similar to humans in anatomy, physiology, and metabolism;[20,22] second, their brains are large enough for easy localization and retrieval of specific brain nuclei; third, the cost of procurement and maintenance is modest compared to that of primates. Moreover, it has been shown that systemic morphine induces tolerance to antinociception in domestic pigs,[22] and a stereotaxic atlas of the pig brain has been published[23] that served as reference to the procurement of the NAc in the present study.

The Lanyu miniature pigs (*Sus scrofa domesticus*) were used as the experimental animals in the present study. They are an indigenous breed from Lanyu Islet (Orchid Island) situated off the coast of southeastern Taiwan. They have a narrow and straight head with long straight snout and small erect ears as well as a small body and coarse dark hair. Two herds of indigenous Lanyu pigs were moved from Lanyu Islet to Taiwan decades ago and have been reared separately by National Taiwan University and the Taitung Animal Propagation Station (TAPS), both for conservation and development of a laboratory pig breed.[24,25] There have been 6 journal articles (all from Taiwan) that used the Lanyu miniature pigs as the experimental animals.[24-29]

In the present study, intrathecal rather than systemic administration of morphine was chosen to induce morphine tolerance in the Lanyu miniature pigs. The intrathecal route theoretically simplifies the pharmacokinetic profile and circumvents the blood-brain barrier, which confounds the central bioavailability of systemic morphine;[30] its drawback, however, is technical difficulty in placing an intrathecal catheter.

1.4 Proteomics

The word "proteome" (*prote*in complement to a gen*ome*) was coined by Wilkins *et al.* in 1994; Wilkins introduced the concept of proteome at the First Siena conference themed "2D electrophoresis: from protein maps to genomes" later in the same year.[31] "Proteome" is the collection of all the proteins expressed by a genome within a particular cell, tissue, organ or organism at a particular time.[32] Proteomics is the study of the proteome in a holistic manner.

The advent of new concepts and development of technology have transformed traditional protein chemistry into modern, highly complex proteomics.[31] In the past decade, there have been tremendous advances in proteomic technologies. Several sophisticated technologies including two-dimensional electrophoresis (2-DE), imaging, mass spectrometry (MS), and bioinformatics make possible the simultaneous separation of hundreds or thousands of peptides/proteins and their further quantification and characterization.[33] A typical proteomic workflow includes:[34]

(a) Sample collection and storage;

(b) Sample preparation, prefractionation and separation (by molecular weight (MW), isoelectric point (pI), or chemical affinity of each protein);

(c) Protein quantification and characterization by MS;

(d) Bioinformatics for identification of proteins or biomarkers.

Clinical proteomics, the application of proteomics techniques to the medical field, aims to identify proteins involved in the pathogenesis of a specific disease state. This approach has the potential to identify biomarkers that allow the diagnosis or treatment of the disease.[33,35,36]

There are inherent limitations in the proteomics approach, however, such as the difficulties in detection of proteins of low abundance, hydrophobic proteins or proteins with extreme pI or MW.[37]

1.5 Two-dimensional difference in gel electrophoresis (2D-DIGE)

A common combination of proteomic technologies for separation and identification of proteins is 2-DE coupled with MS. 2-DE, the long-time standard for protein separation, was first described in 1975.[38] It separates protein mixtures in two steps: in the first dimension (isoelectric focusing (IEF)), proteins are separated according to their pI along a pH gradient generated by a pre-cast immobilized pH gradient (IPG) strip; in the second dimension, proteins are resolved according to their MW.[39] Traditional 2-DE allows simultaneous visualization of large portions of the proteome, but it suffers from the requirement of multiple gels, gel-to-gel variations, poor reproducibility and unreliable quantitative capabilities.[5,40]

In 1997 Ünlü *et al.* first describe 2D-DIGE.[41] 2D-DIGE builds on 2-DE technique but comes with improved accuracy and reproducibility. This technique allows separation of more than one protein extracts on the same 2D gel (multiplexing) thanks to the use of fluorescent dyes (CyDye DIGE fluors) that are spectrally resolvable as well as mass- and charge-matched. Two protein extracts are prelabeled with either Cy3 or Cy5, mixed, and run on a single 2D gel. A third dye Cy2 allows labeling of a reference sample (the pooled internal standard) that are made up of equal amounts of all biological samples in an experiment and co-electrophoresed with Cy3- or Cy5- labeled protein extracts on each 2D gel. The pooled internal standard acts as a baseline for inter-gel comparisons of protein abundance. The proteins are then visualized using a fluorescence scanner; the images are quantified and statistical analysis is done using DeCyder program.[40]

Compared to traditional 2-DE, 2D-DIGE has the following advantages:[40,42,43]

(a) Accurate quantification;

(b) Multiplexing, which translates to less gel-to-gel variations;

(c) Comparable or better sensitivity;

(d) Feasibility of advanced statistics such as multivariate analysis.

With so many advantages, 2D-DIGE has become “the new gold standard for 2-D gel electrophoresis.”[42] But it is not without drawbacks:[5]

(a) Expensive dyes and equipment;

(b) The possibility that proteins with low lysine content will be labeled less efficiently.

第二章　材料與方法

本研究以三至四個月大的蘭嶼迷你猪(Lanyu miniature pigs, *Sus scrofa domesticus*)為實驗動物。嗎啡組與對照組各 6 隻實驗動物。將實驗動物麻醉後，以枕骨大孔穿刺方式將導管放置於每一隻實驗動物的脊髓腔內。

對於嗎啡止痛效果的耐受性的評估，是測量抗傷害感受反應，採用類似 Risdahl 等人發表過的方式，以高強度的光源來產熱，照射實驗動物的體側，測量此熱刺激引發軀幹表皮反射(cutaneous trunci reflex, CTR)所需的延遲時間。為避免對實驗動物造成灼傷，熱刺激以 40 秒為時間上限。

嗎啡組以每天注射兩次鹽酸嗎啡 75 μg/kg 至腦脊髓膜內共 10 天來誘發對止痛效果的耐受性；對照組則以生理食鹽水取代嗎啡。每天測量熱刺激引發軀幹表皮反射所需的延遲時間。在腦脊髓膜內注射的第 11 天，先測量熱刺激引發軀幹表皮反射所需的延遲時間，然後在兩組實驗動物皆注射嗎啡 75 μg/kg 至腦脊髓膜內以進行挑戰試驗(challenge test)，每隔 15 分鐘測量熱刺激引發軀幹表皮反射所需的延遲時間，測量至挑戰試驗後的 120 分鐘為止，以此評估嗎啡挑戰試驗的效果。

隨即將實驗動物麻醉，進行機械換氣，以手術分離頸部動、靜脈之後，靜脈注射氯化鉀使實驗動物安樂死。以冰冷的生理食鹽水灌流其腦部後取出腦部，切成 3 mm 厚的矢狀切片，再將伏隔核挖下，暫存於液態氮中。

分離出各實驗動物的伏隔核蛋白質溶胞產物(lysate)後，先進行二維差異膠體電泳的最小標記(minimal labeling)，之後進行第一維等電點聚焦電泳法(isoelectric focusing, IEF)及第二維 SDS-聚丙烯膠電泳(SDS-polyacrylamide gel electrophoresis)。膠片以 Typhoon 9400 螢光掃描器掃描，以 DeCyder 軟體分析其影像，偵測蛋白質點，並對各蛋白質點進行定量分析，以找出在兩組實驗動物間呈現差異表現的蛋白質點。

將欲鑑定的蛋白質點從膠片上挖出，以胰蛋白酶(trypsin)將蛋白質消化為胜

肽，再以液相層析串聯質譜儀分析，將結果與蛋白質資料庫進行搜尋比對，以鑑定出蛋白質。呈現差異表現的部分蛋白質再以西方墨點法進一步確認。

Chapter 2
MATERIALS AND METHODS

2.1 Overview

The flowchart of the present study is depicted in Figure 1.

2.2 Study approval and guidelines

The study was approved by the Institutional Animal Care and Use Committee of Chi Mei Medical Center, Tainan, Taiwan and followed the guidelines established by the National Science Council of Taiwan.

2.3 Animals

Twelve Lanyu miniature pigs of both genders, weighing 18–22 kg and aged 3–4 months, were purchased from the TAPS, Taiwan Livestock Research Institute, Taitung, Taiwan. Animals were housed in a climate-controlled room (maintained at 25 °C) with natural lighting, *ad libitum* access to water, and twice daily feedings of standard pig chow. Animals were reared individually while allowed nose-to-nose contacts with those in adjacent pens. Animals were allowed a habituation period of at least 7 days before the initiation of the following procedures.

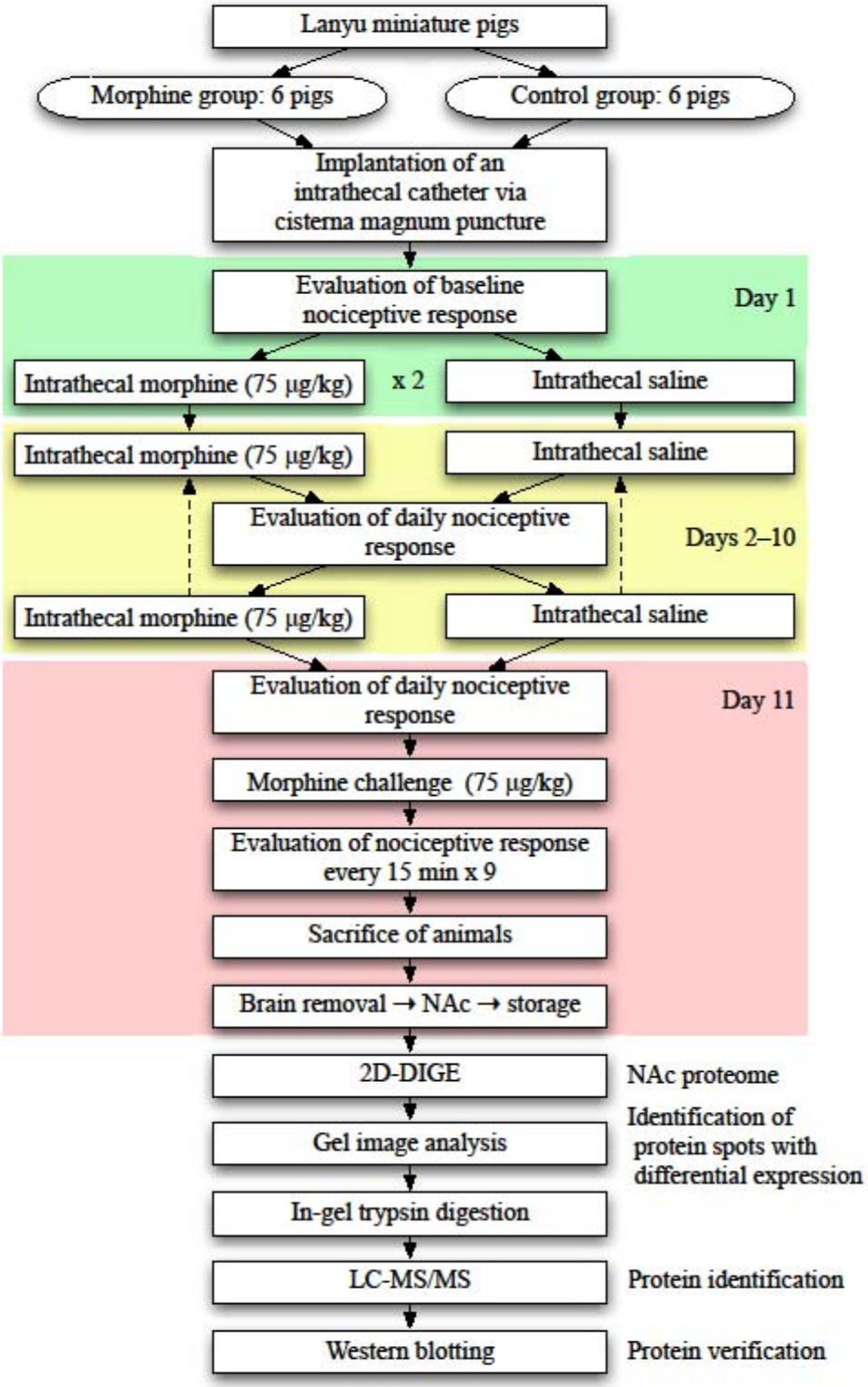

Figure 1 Flowchart.

2.4 Implantation of intrathecal catheters

All of the animals were anesthetized with intramuscular injection of tiletamine-zolazepam (Zoletil 50) 50–100 mg along with atropine 1 mg. Intravenous access was established by cannulating an auricular vein. Animals were placed in the lateral decubitus position with the neck flexed. Cisterna magnum puncture was done under aseptic conditions using a Tuohy epidural needle (Perican 18G × 80 mm; B. Braun, Melsungen, Germany) and loss-of-resistance method carefully to avoid damage to the underlying nervous structures. An epidural catheter (Perifix Standard 20G × 100 cm; B. Braun) was then advanced in the caudad direction through the epidural needle into the subarachnoid space with 8 cm of the epidural catheter past the distal extent of the needle, which was then removed. The epidural catheter was secured with silk sutures and tapes. Throughout the procedure, supplemental tiletamine-zolazepam in combination with thiopental was given intravenously as required. Animals were then allowed a 1-day recovery period.

2.5 Evaluation of morphine analgesic tolerance

To measure nociceptive responses in animals in a pen situation, a portable, high-intensity light-powered thermal stimulating device similar to that described by Risdahl *et al.*[22] was held 24 cm away from the animals to measure the latency time (in seconds) needed for the emitted heat to elicit cutaneous trunci reflex (CTR) in the animals. CTR was measured at the same location on the flank of each animal. A cutoff time of 40 s was used to avoid skin burns of the animals.

Morphine solution for intrathecal injections was prepared by dissolving preservative-free morphine hydrochloride powder (obtained from the National Bureau of Controlled Drugs, Taipei, Taiwan) in normal saline to achieve a concentration of 2 mg ml^{-1}. The solutions were sterile-filtered and stored in colored Eppendorf tubes.

Twelve animals were divided into two groups. On day 1 of intrathecal injection, baseline latency for thermal stimulation to elicit CTR was measured in both groups in the morning before feeding. Then animals in the morphine group (n = 6) were injected morphine 75 μg/kg followed by saline 1 ml intrathecally, whereas those in the control group (n = 6) received an equivalent volume of intrathecal saline instead of morphine.

Intrathecal injection was repeated 8 h later in the afternoon. On days 2–10, morphine and/or saline administration was the same as that on day 1; CTR latency was measured 1 h after intrathecal injection in the morning. On day 11, CTR latency was measured, then morphine 75 μg/kg followed by saline 1 ml was injected intrathecally in both groups for morphine challenge. CTR latency was measured (at 15-min intervals until 120 min after morphine injection) to evaluate the effect of morphine challenge; the area under the curve (AUC) of CTR latency was calculated to evaluate morphine analgesia.[3]

2.6 NAc procurement

Anesthetic procedures were similar to those for implantation of intrathecal catheters, except that animals were orotracheally intubated, paralyzed with intravenous rocuronium, and mechanically ventilated. The intrathecal catheter was removed. Bilateral carotid arteries and jugular veins were surgically exposed to prepare for brain perfusion. Potassium chloride 40–80 mEq was injected into one of the jugular veins until the electrocardiograph showed cessation of cardiac activity. The brain was infused via the carotid artery with 2500–3000 ml of ice-cold normal saline. Animals were extubated, decapitated and the brain removed *en bloc*. The brain was cut into 2 hemispheres on ice and immersed in liquid nitrogen for 2 min to harden it. Each brain hemisphere was cut from the midline laterally into 3-mm sagittal sections with a slicer (Topaz 195; Sirman, Pieve di Curtarolo, Italy). NAc was identified using lateral ventricle and optic chiasma as landmarks,[23] and the dissected NAc tissue was stored in liquid nitrogen awaiting further processing.

2.7 Preparation of NAc protein lysates

Isolated pig NAc tissues were ground under liquid nitrogen in mortar and then transferred to centrifuge tubes containing the appropriate amount of lysis buffer [7 M urea, 2 M thiourea, 4% CHAPS, 1 mM EDTA, 1 mM PMSF, 100 U/ml aprotinin, 100 mM DTT and 1 tablet of *Complete Mini* protease inhibitor cocktail tablet (Roche Diagnostics, Indianapolis, IN, USA) per liter]. The samples were then processed by sample grinding kit (GE Healthcare, Piscataway, NJ, USA) according to the protocol provided by the manufacturer, followed by centrifugation at 14 000 *g* for 20 min. The

supernatant was collected and centrifuged again at 320 000 *g* in an Optima ultracentrifuge (Beckman Coulter, Fullerton, CA, USA). After centrifugation, the supernatant was cleaned with 2-D Clean-Up Kit (GE Healthcare) and the protein pellet was dissolved in rehydration buffer for electrophoresis.

2.8 2D-DIGE

For DIGE minimal labeling, 33 μg of proteins were mixed with 264 pmol CyDye (GE Healthcare) by vortexing and incubated on ice in the dark for 30 min. Proteins extracted from the two animal groups were labeled with either Cy3 or Cy5 using a dye swapping strategy (Table 1) as follows: in gels 1–3, proteins from the control group were labeled with Cy3, and proteins from the morphine group were labeled with Cy5; in gels 4–6, Cy3 and Cy5 were interchanged. To make up 33 μg of the pooled internal standard bulk-labeled by Cy2, 2.75 μg of proteins from each of 12 lysates, which were prepared from 6 pairs of morphine-treated/control pigs, were pooled together. In all three cases the labeled sample was then quenched by the addition of 1 μl 10 mM lysine (Sigma-Aldrich, St. Louis, MO, USA) followed by incubation on ice for a further 10 min. Before the first-dimension IEF, a 33 μg aliquot from each of three labeled mixes (Cy3, Cy5 and Cy2-labeled) were combined with the rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 2% DTT, 0.5% IPG buffer and trace amount of bromophenol blue) that covered the pH 4–7 interval of the IPG strips, to give a final volume of 320 μl. In-gel rehydration of the 18 cm IPG strip (GE Healthcare) with the 320-μl rehydration buffer containing the protein sample was performed at 20 ºC in the dark for 16 h. The proteins were then focused at 20 ºC at 200 V, 500 V, 1000 V, 5000 V and 8000 V with a total of 81 734 Vh using IPGphor electrophoresis unit (GE Healthcare). After IEF, the gel strips were equilibrated in equilibration buffer (0.5 M Tris-HCl (pH 8.8), 6 M urea, 30% glycerol, 2% SDS) containing 1% DTT for 15 min and then in equilibration buffer containing 4% iodoacetamide for a further 30 min. The equilibrated gel was loaded onto the top of a 10% acrylamide gel and sealed with 0.5% agarose. The proteins were separated at 420 V using Bio-Rad Protean IIxi (Bio-Rad, Hercules, CA, USA) until bromophenol blue reached the bottom of the gel.

Table 1 Experimental design.

Gel number	Cy2	Cy3	Cy5
1	Pooled internal standard	Control 1	Morphine 1
2	Pooled internal standard	Control 2	Morphine2
3	Pooled internal standard	Control 3	Morphine 3
4	Pooled internal standard	Morphine4	Control 4
5	Pooled internal standard	Morphine5	Control 5
6	Pooled internal standard	Morphine6	Control 6

2.9 Gel image analysis

The gels were scanned with a Typhoon 9400 fluorescence scanner (GE Healthcare). Image analysis was performed by DeCyder 5.01 program (GE Healthcare). The differential in-gel analysis (DIA) mode of DeCyder was implemented for spot detection and normalization of the control and morphine-treated gel images to the internal standard. After spot detection, the abundance of each spot was shown by normalized volume (Cy3/Cy2 or Cy5/Cy2), represented by the ratio of the control or morphine-treated sample (Cy3 or Cy5) to the internal standard (Cy2). In DIA spot detection, volume < 30 000 was used for spot filtering. Using the DeCyder biological variation analysis (BVA) mode, spot matching and abundance comparison was performed in automatic mode, and the average ratio and Student's *t*-test value for each protein spot were calculated based on all the gel images (morphine : control, $n = 6$ per group). The protein spot matches and differential abundances were confirmed by visualization for all the gels. Moreover, the differentially expressed proteins with statistical significance were further assessed by the three-dimensional spot profiles.

2.10 In-gel digestion

The in-gel digestion was performed as described previously.[35] In brief, the spots excised from the gel were incubated in a solution containing 15 mM of potassium ferricynate and 50 mM sodium thiosulfate until the brownish color disappeared. The destained gel piece was washed with 25 mM ammonium bicarbonate for 10 min and

then with 25 mM ammonium bicarbonate/50% (v/v) acetonitrile for 10 min. After drying in SpeedVac (Thermo Savant, Milford, MA, USA), the gel was incubated with 50 μl of 2% (v/v) 2-mercaptoethanol in darkness for 20 min, an equal volume of 10% (v/v) vinylpyridine in 25 mM ammonium bicarbonate/50% (v/v) acetonitrile was added, and the gel was incubated further for 20 min. Then the gel was washed three times with 25 mM ammonium bicarbonate and dehydrated in 25 mM ammonium bicarbonate/50% (v/v) acetonitrile. The gel was dried and treated with 50 ng of modified trypsin (GE Healthcare) in 100 μl of 25 mM ammonium bicarbonate at 37 °C overnight. The supernatant was collected after digestion and the gel was extracted with 200 μl of 0.1% (v/v) formic acid. The extracts were combined and dried in SpeedVac and resuspended in 0.1% (v/v) formic acid immediately for MS analysis or stored at –20 °C until use.

2.11 Protein identification analysis via LC-MS/MS

The protein digest was analyzed in LTQ-Orbitrap hybrid tandem mass spectrometer (Thermo Fisher, Waltham, Massachusetts, USA) in-line coupled with Agilent 1200 nanoflow HPLC system equipped with LC Packing C18 PepMap 100 (length: 5 mm; internal diameter: 300 μm; bead size: 5 μm) as the trap column and Agilent ZORBAX XDB-C18 (length: 50 mm; internal diameter: 75 μm; bead size: 3.5 μm) as the separating column (Agilent Technologies, Santa Clara, CA, USA). File Converter in Xcalibur 2.0SR package (Thermo Fisher) and an in-house program were used to extract the MS/MS information as well as to compute the charge and mass for each analyzed peptide. TurboSequest program (ver. 27, rev. 11) was then used to search the best matched peptides from a non-redundant (NR) protein database whose FASTA sequences were downloaded from National Center for Biotechnology Information (NCBI, ftp://ftp.ncifcrf.gov/pub/nonredun/). While only the tryptic peptides with $\leqq$ 2 missed cuts were considered, the mass ranges during the database search were 1 and 3.5 *m/z* for fragment and precursor ions, respectively. The protein identities were verified only when there were at least two peptides matched and both search results had high Xcorr (i.e. $\geqq$ 2.0 for doubly charged peptides and $\geqq$ 3.0 for triply charged ones) and with minimal differences between observed and hypothetical masses (i.e. ΔM <10 ppm).

2.12 Western blotting

After isolated pig NAc tissues were ground under liquid nitrogen in mortar, the proteins were lysed in the sample buffer (0.1 M Tris, pH 6.8, 2% SDS, 0.2% β-mercaptoethanol, 10% glycerol and 0.0016% bromophenol blue). Total cell lysates (50 μg of protein) were separated using 10% acrylamide gel electrophoresis and transferred onto the PVDF membrane (Stratagene, La Jolla, CA, USA). The membrane was blotted with primary antibodies overnight followed by incubation with horseradish peroxidase-conjugated secondary antibodies (1:15 000) and visualized using chemiluminescence (GE Healthcare). Primary antibodies against internexin neuronal intermediate filament protein α (α-internexin), creatine kinase B-type (CK-B) and elongation factor tu (EF-Tu) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA; α-internexin (2E3) (Ref. sc-58478), creatine kinase-B (N-20) (Ref. sc-15157) and EF-Tu (CBP-KK1) (Ref. sc-21758)). Primary antibodies against calcineurin catalytic subunit (CaN A) were from Cell Signaling Technology (Danvers, MA, USA; pan-calcineurin A antibody #2614). Primary antibodies against heat shock 60kDa protein (hsp60) were from Calbiochem (San Diego, CA, USA; anti-hsp60 mouse mAb (LK-1)).

2.13 Statistical analysis

Student's *t*-test was used in the DeCyder BVA mode to compare the normalized abundance of matched protein spots between morphine and control groups. Otherwise, SPSS 17 for Windows (SPSS Inc., Chicago, IL, USA) was used for data analysis; comparisons between morphine and control groups were performed using the Mann-Whitney U test. Data were expressed as mean ± standard error of the mean. $P < 0.05$ was considered as significant.

第三章　結果

以蘭嶼迷你猪為實驗動物，每天注射兩次嗎啡 75 μg/kg 至嗎啡組實驗動物的腦脊髓膜內共 10 天，可誘發嗎啡組實驗動物對止痛效果的耐受性。在腦脊髓膜內注射的第 2 至 6 天，嗎啡可顯著延長熱刺激引發軀幹表皮反射所需的延遲時間，但此止痛效果無法持續到第 7 至 11 天。在腦脊髓膜內注射的第 11 天進行嗎啡挑戰試驗，由嗎啡注射後 120 分鐘內每隔 15 分鐘所測量的軀幹表皮反射延遲時間來計算曲線下面積(area under the curve, AUC)以評估嗎啡的止痛效果，同樣顯示嗎啡的止痛效果在嗎啡組被顯著降低($P = 0.004$)。由此可證明嗎啡組實驗動物確實已發生嗎啡耐受性。

為探索嗎啡耐受性如何改變伏隔核的蛋白質表現，將嗎啡組與對照組的伏隔核蛋白質體以二維差異膠體電泳的研究方式進行比較。針對電泳膠片上的蛋白質點以 DeCyder 軟體進行統計分析，發現其中 22 個蛋白質點的含量在兩組間呈現顯著差異($P < 0.05$)。在這 22 個蛋白質點之中，7 個蛋白質點在嗎啡組的表現量增加，15 個蛋白質點在嗎啡組的表現量減少。以液相層析串聯質譜儀加上資料庫比對來鑑定上述的蛋白質點，發現在嗎啡組中表現量增加的 7 個蛋白質點被鑑定為 7 個蛋白質：KIF1A head-microtubule complex, chain A、soluble epoxide hydrolase、selenium-binding protein 1、eukaryotic elongation factor 1 γ-like protein、phytanoyl-CoA 2-hydroxylase interacting protein-like isoform 2、guanine nucleotide binding protein α、GBI1；在嗎啡組中表現量減少的 15 個蛋白質點被鑑定為 11 個蛋白質：mitofilin、heat shock 60kDa protein (hsp60) isoform 1、internexin neuronal intermediate filament protein α (α-internexin)、calcineurin catalytic subunit (CaN A) δ isoform、ATP synthase subunit β、glial fibrillary acidic protein、ubiquinol-cytochrome c reductase、elongation factor tu (EF-Tu)、creatine kinase (CK)或 creatine kinase B-type (CK-B)、succinyl-CoA ligase subunit β、antioxidant protein isoform 1。這些被鑑定出來的蛋白質，功能與細胞骨架、細胞訊息、代謝、蛋白質轉譯及蛋白質摺疊有關。

最後以西方墨點法評估 α-internexin、CaN A、CK-B、EF-Tu、hsp60 在伏隔核

的表現量，發現這五個蛋白質在嗎啡組實驗動物的表現量大多是減少的，與二維差異膠體電泳的結果一致。

Chapter 3

RESUTLS

3.1 Development of morphine tolerance

Morphine analgesic tolerance was demonstrated in Lanyu miniature pigs administered intrathecal morphine 75 μg/kg twice daily for 10 days. Morphine increased the latencies for thermal stimulation to elicit CTR on days 2–6 of morphine administration, but this analgesic effect did not persist to days 7–11 (Figure 2a). Moreover, on day 11 of intrathecal drug administration, the analgesic effect of morphine challenge, as represented by AUC of time course (Figure 2b), was significantly attenuated in the morphine group (AUC = 886.43 ± 28.18) compared to the control group (AUC = 3177.06 ± 84.04, $P = 0.004$).

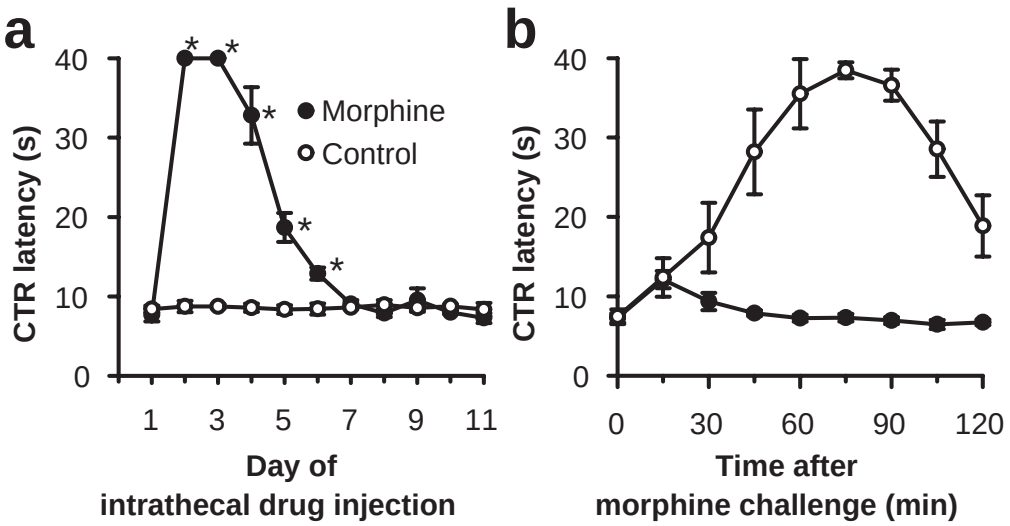

Figure 2 Morphine analgesic tolerance in pigs administered intrathecal morphine 75 μg/kg twice daily for 10 days. Nociceptive response was measured by the latency for thermal stimulation to elicit cutaneous trunci reflex (CTR). A cutoff time of 40 s was used. Latencies were expressed as mean ± standard error of the mean. (a) Time course of nociceptive responses in morphine (n = 6) vs. control (n = 6) groups. (b) Time course of analgesia effected by challenge with intrathecal morphine 75 μg/kg on day 11. The curves were used to calculate the area under the curve (AUC), which represented morphine analgesic activity. *P < 0.05 compared with control group.

3.2 2D-DIGE of NAc

To elucidate how morphine analgesic tolerance changes the protein expression profile in the NAc, the proteome maps of the NAc from morphine-treated pigs and controls were compared using the 2D-DIGE proteomics strategy to measure the alterations in the abundance levels of proteins. These dysregulated proteins may be associated with the molecular mechanisms of morphine analgesic tolerance. Figure 3a illustrated the representative 2D-DIGE proteome maps of morphine-treated animals and controls. The proteins were well separated in the 18 cm gel with pI range of 4–7. On average each gel resolved ~750 protein spots and all the protein spots were subjected to statistical analyses. Quantification and comparison of each protein spot detected on 2D-DIGE gels demonstrated that most of the protein spots were quantitatively similar, but 22 protein spots were differentially expressed between the two animal groups (P <

0.05; Student's *t*-test) with a magnitude near or higher than 1.2-fold (Figure 3b and Table 2). The magnitude of differential expression ranged from –1.5 (spot 321) to +1.69 (spot 338). Of the 22 differentially expressed proteins, 7 proteins were observed to be over-expressed in the morphine group while 15 proteins were under-expressed. The inter- and intra-pair variability for the de-regulated protein spots and corresponding three dimensional images were depicted in Figure 4.

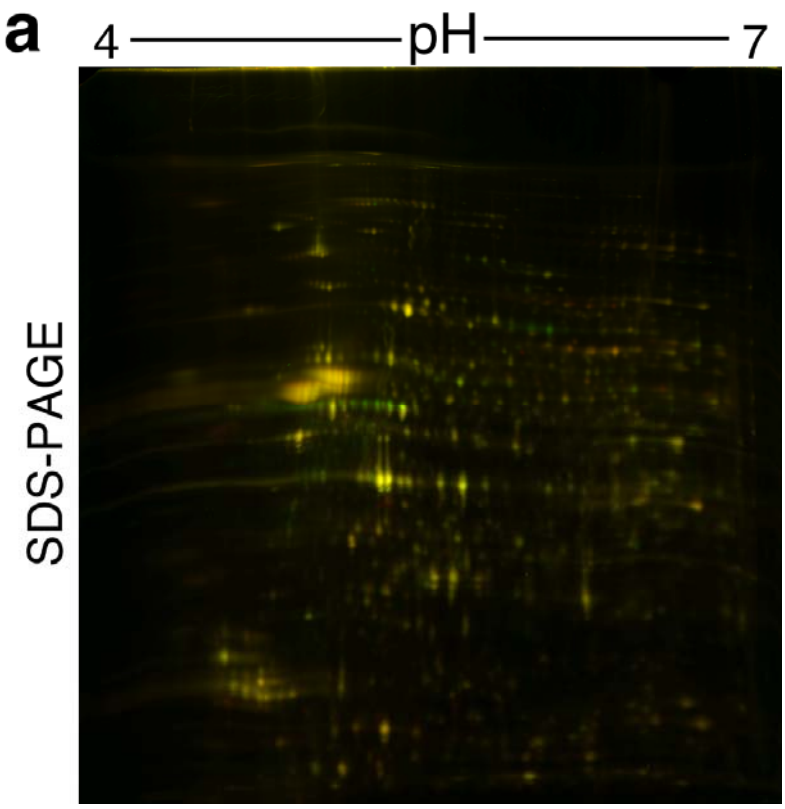

Figure 3 Comparison of nucleus accumbens (NAc) proteome between morphine and control groups by 2D-DIGE. NAc proteins were minimally labeled with CyDye (control/morphine groups with Cy3/Cy5, pooled internal standard with Cy2), separated by isoelectric focusing (IEF) in the first dimension using 18-cm pH 4–7 gel strip as well as by molecular weight using SDS-PAGE in the second dimension. The resultant gel images were captured with a Typhoon imager and analyzed by DeCyder program. (a) A representative overlaid 2D-DIGE image of NAc proteins. (b) Areas of the same gel with proteins of interest boxed and their enlarged counterparts shown to the far right (A–E: control group; A*–E*: morphine group).

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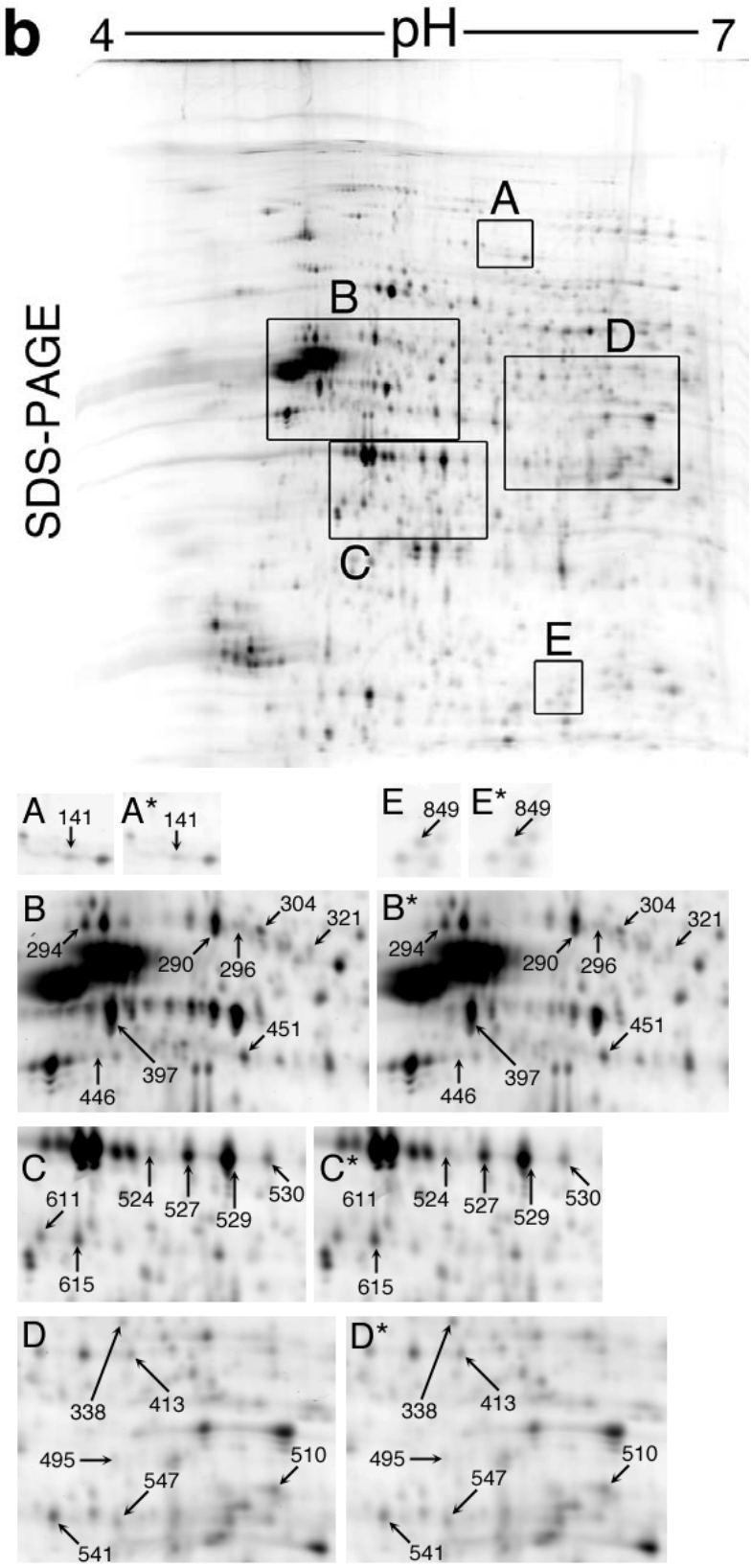

Figure 3 (continued)

Table 2 Proteins of differential expression identified by LC-MS/MS followed by database interrogation.

Up-regulated in the morphine group								
Spot number	*Protein I.D.*	*Accession number*	*Experimental Mr (kDa)/pI*	*Theoretical Mr (kDa)/pI*	*Coverage (%)*	*Matched peptide number*	*Student's t-test (P-value)*	*Average ratio*
294	Chain A, KIF1A head-microtubule complex structure in AMPPNP-form	gi:118137996	61/4.81	50.07/4.91	16.85	3	0.024	+1.14
338	Soluble epoxide hydrolase [*Sus scrofa*]	gi:45551399	57/5.96	62.77/6.06	21.98	6	0.022	+1.69
413	PREDICTED: similar to selenium-binding protein 1 [*Sus scrofa*]	gi:194036227	52/5.97	52.53/6.17	7.42	2	0.0069	+1.17
495	Eukaryotic elongation factor 1 γ-like protein [*Sus scrofa*]	gi:19111049	45/5.94	49.62/6.16	16.17	7	0.0060	+1.25
547	PREDICTED: phytanoyl-CoA 2-hydroxylase interacting protein-like isoform 2 [*Sus scrofa*]	gi:194042683	40/5.95	37.55/6.5	17.88	4	0.034	+1.13
611	*Homo sapiens* guanine nucleotide binding protein α	gi:20147681	36/4.98	40.05/5.34	15.25	5	0.029	+1.35
615	GBI1 protein [*Sus scrofa*]	gi:178056266	36/5.07	40.52/5.22	12.68	4	0.020	+1.35

(continued on next page)

Table 2 (continued)

Down-regulated in the morphine group								
Spot number	*Protein I.D.*	*Accession number*	*Experimental Mr (kDa)/pI*	*Theoretical Mr (kDa)/pI*	*Coverage (%)*	*Matched peptide number*	*Student's t-test (P-value)*	*Average ratio*
141	*Homo sapiens* mitofilin mRNA, partial cds.	gi:8131893	80/5.71	83.7/6.08 (full length)	10	4	0.021	−1.19
290	PREDICTED: heat shock 60kDa protein isoform 1 [*Sus scrofa*]	gi:194044029	61/5.12	60.91/5.71	68.24	26	0.022	−1.32
296	PREDICTED: similar to internexin neuronal intermediate filament protein α [*Sus scrofa*]	gi:194041957	61/5.18	55.39/5.35	25.45	6	0.036	−1.23
304	PREDICTED: similar to internexin neuronal intermediate filament protein α [*Sus scrofa*]	gi:194041957	60/5.23	55.39/5.35	39.28	12	0.041	−1.26
321	Calcineurin catalytic subunit δ isoform [*Sus scrofa*]	gi:14209665	58/5.35	57.64/5.87	24.46	8	0.0040	−1.5
397	PREDICTED: similar to ATP synthase subunit β, mitochondrial [*Sus scrofa*]	gi:194037554	52/4.87	56.34/5.15	76.14	28	0.022	−1.21

(continued on next page)

Table 2 (continued)

Spot number	*Protein I.D.*	*Accession number*	*Experimental Mr (kDa)/pI*	*Theoretical Mr (kDa)/pI*	*Coverage (%)*	*Matched peptide number*	*Student's* t-*test* (P-*value)*	*Average ratio*
446	Glial fibrillary acidic protein [*Sus scrofa*] (GFAP) fragment	gi:29335683	52/4.87	49.9/5.42 (Human GFAP)	18.64	3	0.032	−1.23
451	PREDICTED: similar to ubiquinol-cytochrome c reductase [*Sus scrofa*]	gi:194041191	49/5.19	52.7/5.76	44.58	13	0.018	−1.19
510	*Bos taurus* (bovine) elongation factor tu, mitochondrial precursor (EF-Tu)	gi:1352352	42/6.32	49.4/6.72	17.04	4	0.041	−1.29
524	*Bos taurus* (bovine) retinal creatine kinase	gi:13096153	41/5.25	42.6/5.48	17.37	2	0.026	−1.27
527	Pig creatine kinase B-type (B-CK) fragment	gi:3183054	41/5.33	42.6/5.34 (human B-CK full length)	28.43	2	0.0034	−1.31
529	*Canis familiaris* (dog) creatine kinase B-type	gi:125292	41/5.43	42.70/5.47	35.96	10	0.015	−1.38
530	*Canis familiaris* (dog) creatine kinase B-type	gi:125292	41/5.52	42.70/5.47	21.52	4	0.016	−1.36

(continued on next page)

Table 2 (continued)

Spot number	*Protein I.D.*	*Accession number*	*Experimental Mr (kDa)/pI*	*Theoretical Mr (kDa)/pI*	*Coverage (%)*	*Matched peptide number*	*Student's t-test (P-value)*	*Average ratio*
541	Pig succinyl-CoA ligase [ADP-forming] subunit β, mitochondrial precursor	gi:21263966	41/5.79	46.26/6.08	37.65	8	0.049	−1.21
849	PREDICTED: similar to antioxidant protein isoform 1 [*Sus scrofa*]	gi:194042132	19/5.95	28.47/7.20	12.64	2	0.048	−1.37

(Mr: molecular weight)

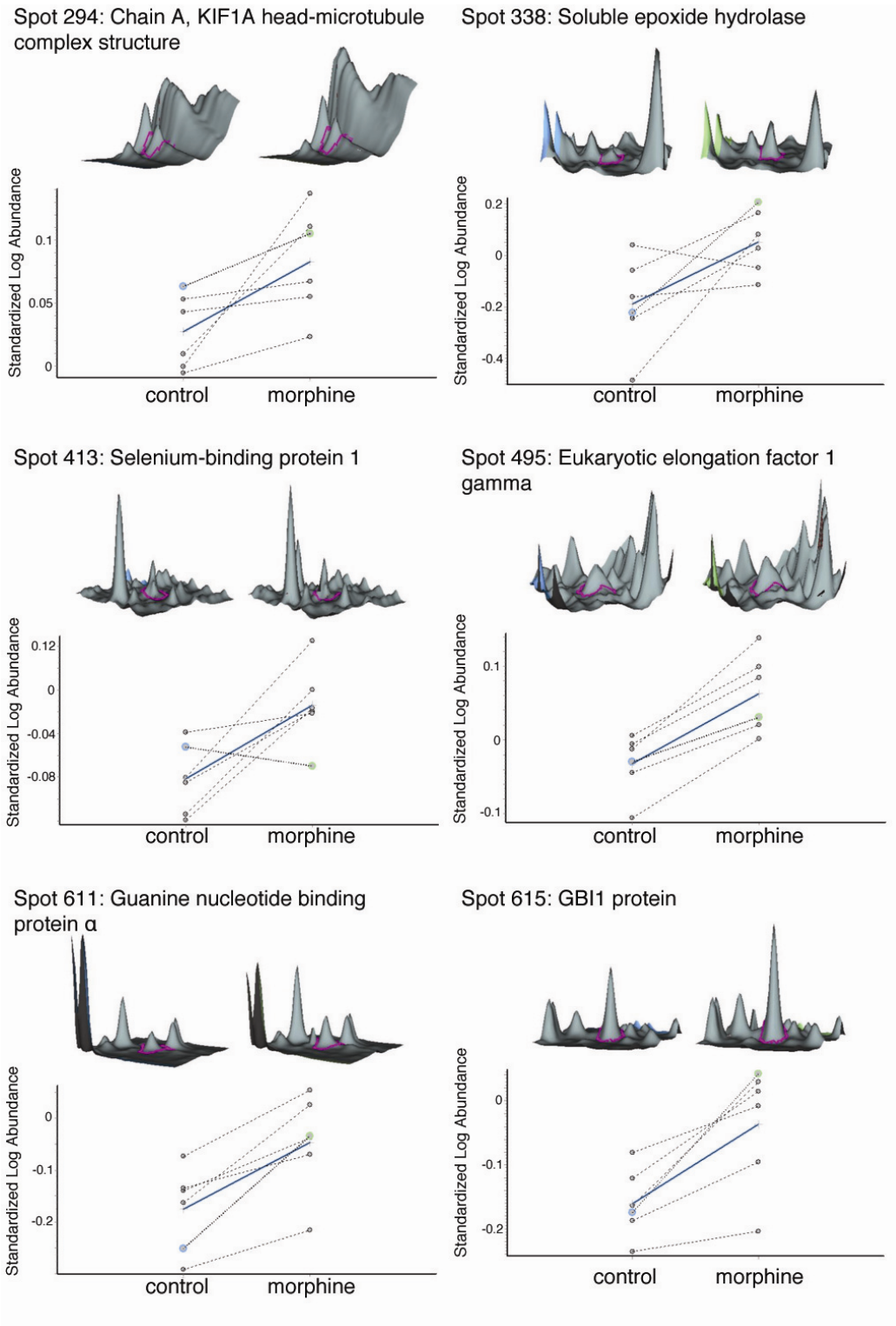

Figure 4 Representative proteins of differential expression as determined by 2D-DIGE and analyzed with DeCyder program. Each panel shows the standardized log abundance of a protein spot in each animal in control or morphine group. At the top of each panel are a pair of representational three-dimensional images of the protein spot.

(continued on next page)

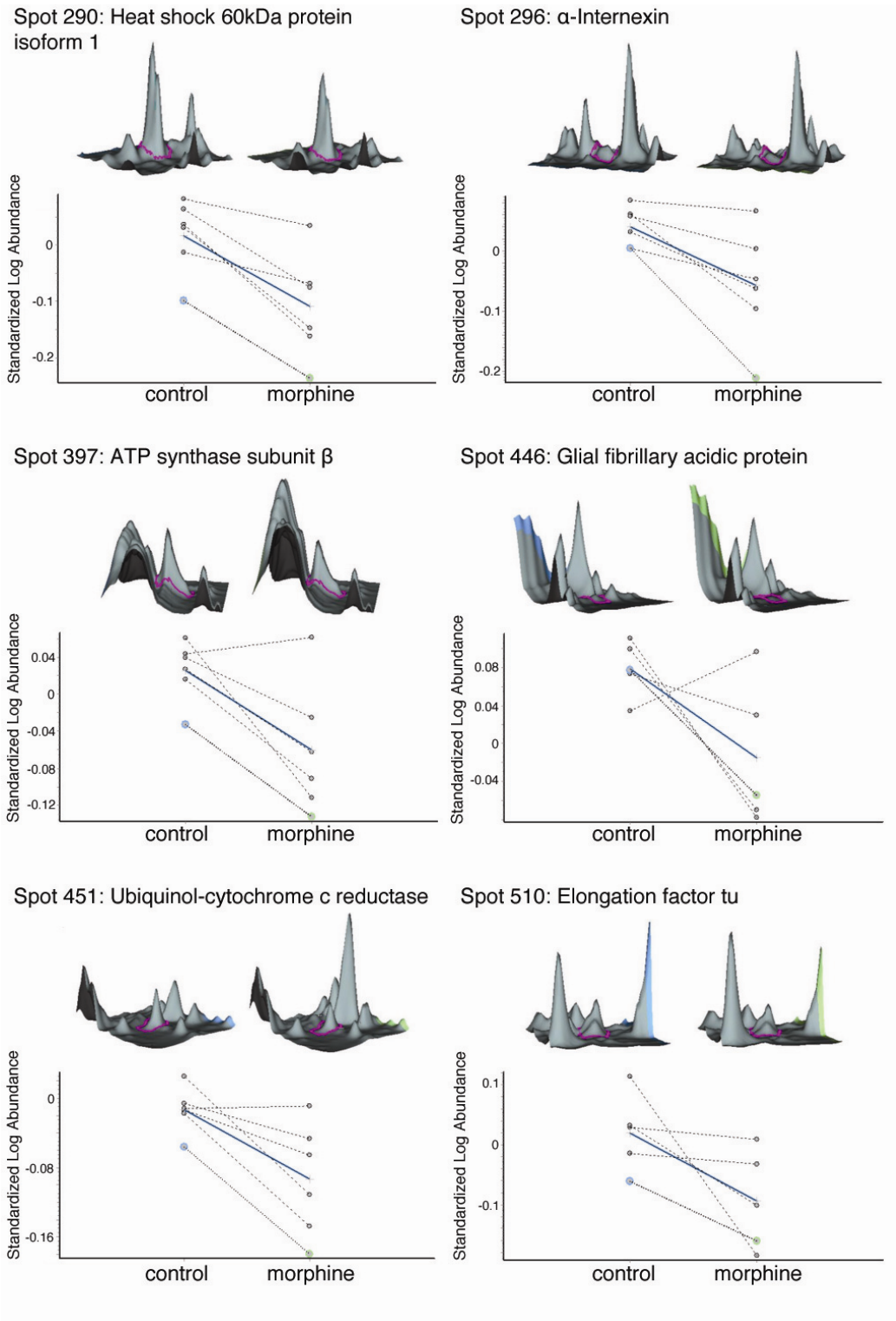

Figure 4 (continued)

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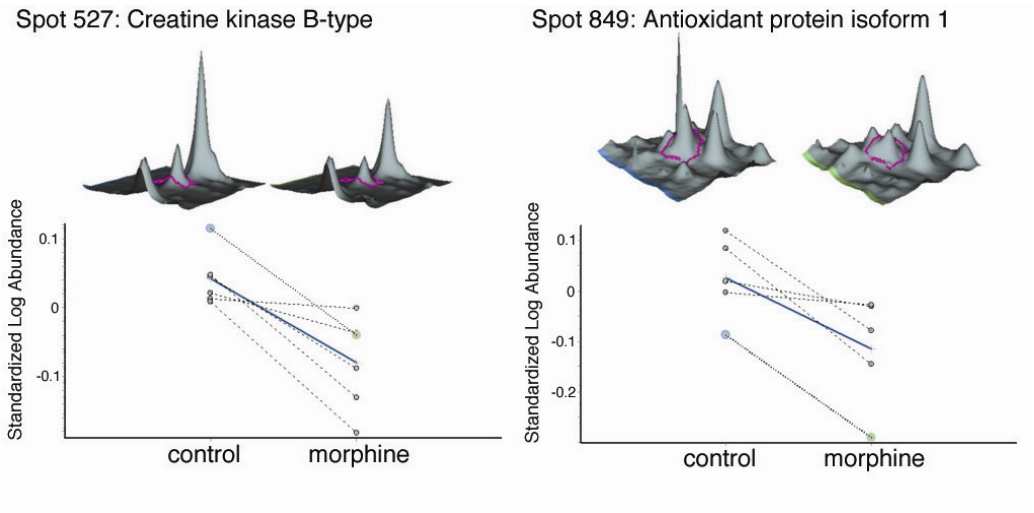

Figure 4 (continued)

3.3 Identification of differentially expressed proteins

After analyzing the proteome maps, peptides were extracted from each differentially expressed protein spot by in-gel tryptic digestion and proteins were identified using LC-MS/MS. The MS/MS information for each peptide was searched against NR database of NCBI. The results of spectrometric analyses are summarized in Table 2. The experimental MW and pI of most protein spots were similar to the theoretical values. Seven protein spots exhibited statistically significant up-regulation in morphine-treated pigs and were characterized as 7 proteins, while 15 protein spots were significantly decreased in morphine-treated pigs and were identified as 11 proteins. The identified de-regulated proteins are involved in cytoskeletal functions, cell signaling, metabolism, protein translation and protein folding.

3.4 Confirmation of differentially expressed proteins by Western blotting

De-regulation of cytoskeletons, hsp and CK has been implicated in chronic cocaine use;[44,45] CaN-mediated pathway has been reported to be involved in learning, memory and morphine abuse.[46-49] Based upon the above observations, to confirm our speculation on the 2D-DIGE results, Western blotting was employed to assess the expression of α-internexin, CaN A, CK-B, EF-Tu and hsp60. Consistent with the proteomics results, these five proteins were discovered to be down-regulated in most morphine-treated pigs by Western blotting (Figure 5).

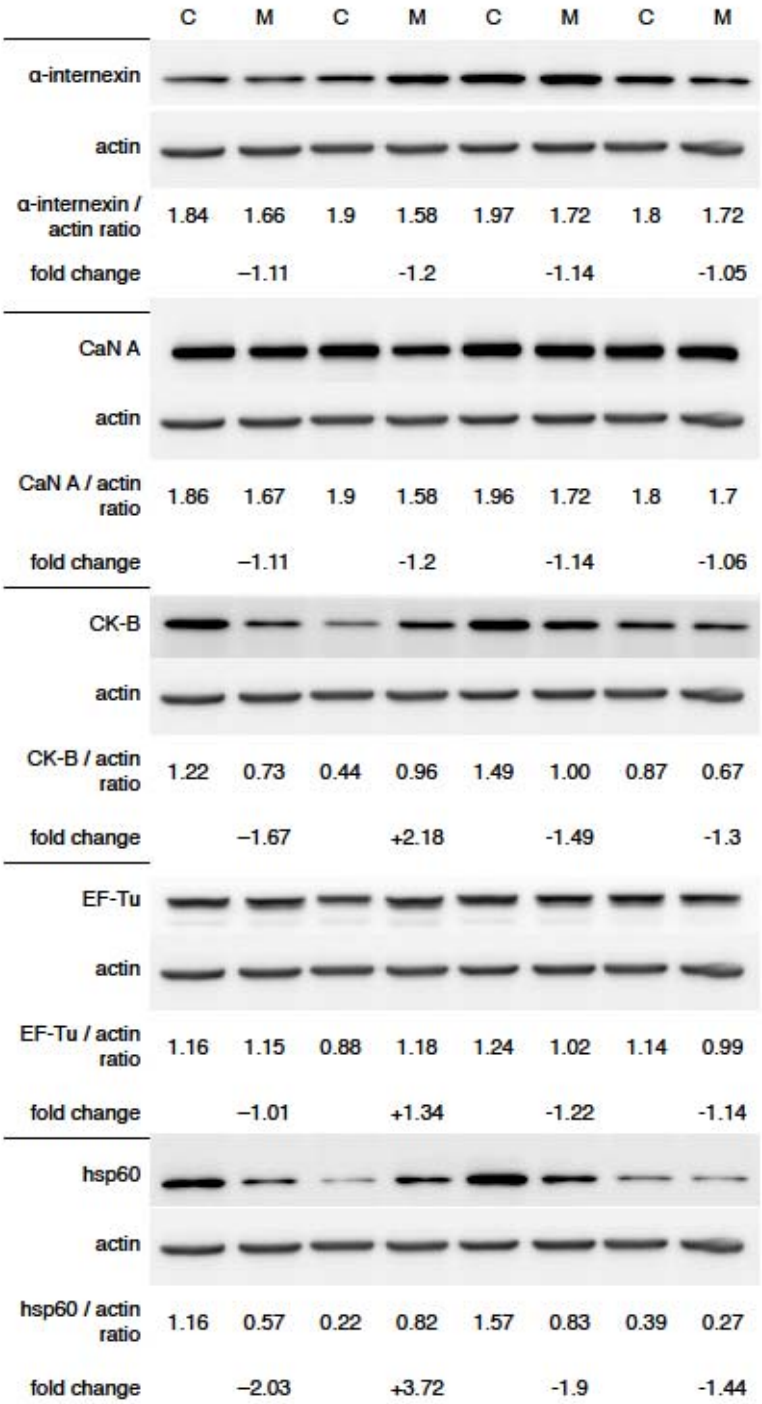

Figure 5 Western blotting of 5 identified proteins of differential expression between morphine and control groups. C, control; M, morphine; CaN A, calcineurin catalytic subunit; CK-B, creatine kinase B-type; EF-Tu, elongation factor tu; hsp60, heat shock 60kDa protein.

第四章　討論

本研究以蛋白質體學的研究方式發現豬的伏隔核中十幾個與嗎啡耐受性可能有關的蛋白質。就目前所知，這是探索與嗎啡耐受性相關的豬的伏隔核蛋白質體的第一個研究。本研究所發現的呈現差異表現的蛋白質可以分類為結構、細胞訊息、代謝、粒線體及其他。

結構：α-internexin (neurofilament-66)及 glial fibrillary acidic protein 在嗎啡組實驗動物的伏隔核中皆呈現表現量減少。二者皆為中間絲蛋白質(intermediate filament proteins)，為細胞骨架(cytoskeleton)的成分。前人研究發現長期給予大鼠嗎啡可減少伏隔核神經元的樹突顯微結構的複雜程度，且可能會減慢從腹側被蓋區(ventral tegmental area)到伏隔核的軸突輸送(axonal transport)。本研究的結果顯示嗎啡耐受性對於伏隔核的細胞骨架具有特定影響。

細胞訊息：包括 CaN A δ isoform 及 KIF1A。前者藉由將與神經元功能有關的蛋白質去磷酸化(dephosphorylation)而調節神經可塑性(neuroplasticity)；後者為細胞內輸送蛋白，負責將突觸小泡前驅物(synaptic vesicle precursors)延著軸突向突觸末梢(synaptic terminals)輸送。

代謝：包括 soluble epoxide hydrolase、eukaryotic elongation factor 1 γ 及 selenium-binding protein 1，三者在嗎啡組實驗動物的伏隔核中皆呈現表現量增加。Soluble epoxide hydrolase 會代謝環氧二十碳三烯酸(epoxyeicosatrienoic acids)，後者具有止痛及神經保護作用。Eukaryotic elongation factor 1 在生物體內的蛋白質合成過程中扮演重要角色；eukaryotic elongation factor 1 γ 可能是藉由對多巴胺 D3 接受器(dopamine D3 receptor)的作用而促成嗎啡耐受性。

粒線體：antioxidant protein isoform 1 及 ubiquinol-cytochrome c reductase 在嗎啡組實驗動物的伏隔核中皆呈現表現量減少。前者的表現量減少可能使伏隔核易受到氧化傷害(oxidative damage)。

其他：hsp60 1 (chaperonin) isoform 1 及 CK-B 在嗎啡組實驗動物的伏隔核中皆

呈現表現量減少。前者能將新生胜肽摺疊成具有功能的立體形態，故其表現量減少可能造成新生蛋白質的缺陷。CK 在腦細胞中提供能量的緩衝。前人報告過鴉片類藥物會造成腦部代謝的全面降低；對古柯鹼成癮的恒河猴，伏隔核的 CK 表現量會減少。

總而言之，本研究證明在神經蛋白質體學(neuroproteomics)領域以豬為實驗動物是可行的。本研究的發現可能有助於釐清與鴉片類藥物耐受性有關的分子機制之間的複雜互動，並提供了未來研究鴉片類藥物耐受性的診斷與治療時可用的蛋白質標的。

Chapter 4
DISCUSSION

The present study was undertaken to induce antinociceptive tolerance in Lanyu miniature pigs with intrathecal morphine and then profile their NAc proteome as a function of morphine tolerance using proteomics strategy. We demonstrated that morphine tolerance in pigs was successfully induced with the aforementioned protocol. Moreover, 2D-DIGE followed by gel image analysis and LC-MS/MS identification of differentially expressed proteins provided unbiased, sensitive and comprehensive assessment of the NAc proteome as related to morphine tolerance. To our knowledge, this is the first report investigating the porcine NAc proteome associated with morphine tolerance. The differentially expressed proteins thus identified are classified here into structural, cell signaling, metabolism, mitochondrial or miscellaneous.

4.1 Differentially expressed proteins: structural

Two intermediate filament (IF) proteins, α-internexin (–1.23, –1.26) and glial fibrillary acidic protein (GFAP; –1.23), were identified in the present study as differentially expressed structural proteins in the NAc associated with morphine tolerance. These findings indicate that morphine tolerance has specific effects on the NAc cytoskeleton. In the present study, two adjacent protein spots (296 and 304) with similar average ratios on the 2D-DIGE gels were identified by MS as α-internexin; these may represent different post-translational modifications.

α-Internexin (or neurofilament-66), a type IV IF protein found in the CNS neurons,[50-53] may act as the scaffold of neuronal cytoskeleton.[54] It enhances neurite outgrowth and up-regulates other neurofilaments (NF).[55,56] α-Internexin and the NF triplet proteins (NF-L, NF-M and NF-H for low, medium and high MW) are functionally interdependent. By co-assembly with the NF triplet proteins, α-internexin facilitates the axonal transport of NF assemblies.[57]

The effects of drugs of abuse on the expression of NF proteins (including

α-internexin) in the MDS have been investigated. Chronic morphine and cocaine treatments in rats result in unchanged and decreased α-internexin immunoreactivity, respectively, in the VTA.[58] In the prefrontal cortex of human opioid addicts, the NF triplet proteins are decreased, but the level of α-internexin remains unchanged.[59] In the present study α-internexin was down-regulated in the NAc of morphine-tolerant pigs. The inconsistency between these studies may represent regional/species specificity of NF regulation by drugs of abuse or different degrees of tolerance to drugs of abuse.[58-60] Furthermore, chronic morphine in rats alters the structure of the NAc neurons, as reflected by decreased complexity and number of dendritic microstructure.[18] Since chronic morphine administration in rats possibly slows down axonal transport from the VTA to the NAc,[61] our finding may imply impaired axonal transport in the VTA-to-NAc pathway as a result of morphine tolerance. The resultant structural/functional alterations of the NAc neurons may decrease downstream signal transduction,[60] impair the normal functions of the MDS[58] and contribute to the development of morphine tolerance. This is consistent with the concept that chronic opioid exposure may induce neural injury.[6]

GFAP is a type III IF protein expressed in astrocytes and other glia.[62] It is commonly used as an astroglial cell marker such that its expression is used to represent astroglial activities. Decreased levels of GFAP have been reported in the frontal cortex of individuals with depression, schizophrenia and bipolar disorder,[63] but the exact mechanisms remain obscure. On the other hand, GFAP expression is considered a marker of neural injury as well as opioid tolerance in the CNS.[62,64] It was first shown in the past decade that chronic morphine treatment in rats induces glial activation (with concomitant GFAP up-regulation) in the spinal cord and brain.[65] Thereafter an astroglial component to the pathophysiology of unwanted effects of chronic opioid exposure was recognized, and activated glial cells are deemed to counter-regulate opioid analgesia and play a central role in creating morphine tolerance.[66] GFAP up-regulation was also discovered in the NAc of rhesus monkeys following cocaine self-administration.[45] In the present study, however, GFAP was down-regulated in the NAc of morphine-tolerant pigs. It has been shown that morphine inhibits murine astroglial growth *in vitro*;[67] besides, CNS astroglial cells may respond to drugs of abuse in a time-related manner. In mice receiving consecutive daily administration of cocaine, up-regulation of GFAP in the dentate gyrus was noted after 7, but not 14, days

of cocaine administration; shrunken and less branched astrocytes observed after 14 daily administration of cocaine may signify neurotoxicity induced by cocaine.[68] Likewise, in a rat model of withdrawal from cocaine, increased GFAP expression in the NAc occurred only following a 3-week withdrawal period but not following shorter withdrawal periods (24 h or 1 week).[69] Moreover, discrepancy in GFAP expression between the present and other studies may also arise from differences in the route or protocol (intermittent vs. continuous) of morphine administration,[70,71] species/strains of animals,[70,72] brain regions[59,69] or confounding by GFAP isoforms or post-translational modifications.[37] Since GFAP has a role on maintenance of myelination,[73] down-regulated GFAP in the NAc may also contribute to injury in the MDS.

4.2 Differentially expressed proteins: cell signaling

In the present study, CaN catalytic subunit δ isoform (–1.5) and chain A, KIF1A head-microtubule complex structure in AMPPNP-form (+1.14) were identified as differentially expressed proteins responsible for cell signaling in the NAc related to morphine tolerance.

CaN, also known as protein phosphatase 3 (PPP3, formerly protein phosphatase 2B), is a calcium- and calmodulin-dependent serine/threonine protein phosphatase regulating many calcium-mediated intracellular signaling processes. CaN has a unique heterodimer structure composed of the CaN A catalytic subunit (PPP3C) and the CaN B regulatory subunit (PPP3R).[74] CaN A α isoform (i.e. CaN catalytic subunit δ isoform) is the predominant CaN A in the brain.[75] The NAc is a part of striatum, which is one of the areas with the highest level of CaN in rat brain.[76,77] CaN plays a critical role in the modulation of neuroplasticity[78] by dephosphorylation of various proteins that regulate neuronal function/excitability and thus signal transduction. Since CaN is a phosphatase, CaN down-regulation may be expected to stimulate downstream neuronal signaling. Besides, one of the best-known adaptations to drugs of abuse is the up-regulation of the cAMP pathway, which activates the transcription factor CREB.[8] CaN acts as a selective filter for stimulation from NMDA receptors, which are a member of an anti-opioidergic system,[1,3] so that only stimulus that is strong enough results in the inactivation of CaN and prolonged phosphorylation of CREB.[78]

Therefore down-regulated CaN, as revealed in the present study, may contribute to the development of morphine tolerance.

CaN and various drugs of abuse modulate the phosphorylation of DARPP-32 (dopamine- and cAMP-regulated phosphoprotein, 32 kDa), a regulator of dopaminergic neurotransmission.[79] Phosphorylated DARPP-32 inhibits protein phosphatase 1 (PP1), which controls various neurotransmitter receptors, ion channels, and transcription factors. In striatal slices of mice that lack DARPP-32, there is a decreased response to drugs of abuse.[80] Thus down-regulated CaN may promote drug abuse phenomenon by virtue of altered regulation of the DARPP-32/PP1 cascade. On the other hand, drug addiction and the process of learning and memory may have common underlying molecular mechanisms, intracellular signaling cascades and similar changes in synaptic plasticity.[81] The aforementioned regulations by CaN may be related to learning and memory:[46] first, memory formation is associated with reduced hippocampal CaN activity;[48] second, CaN inhibition in the amygdala was shown to control the establishment of long-lasting emotional memory;[49] and third, the reinforcing effects of morphine are impaired in transgenic mice overexpressing CaN in the hippocampus.[47] Since the NAc modulates the robustness of memories encoded in the hippocampus,[81] a decreased CaN level in the NAc may play a role in the initiation and persistence of drug addiction.

Kinesin superfamily proteins (KIFs) are ATP-driven intracellular transport proteins ("motor proteins") that carry macromolecules, vesicles and organelles along the microtubule "tracks."[82] KIF1A is a brain-specific member of kinesin 3[83] and participates in anterograde transport of synaptic vesicle precursors (SVP) along axons toward synaptic terminals.[84] KIF1A-mediated axonal transport is vital in the viability and function of neurons.[85] To our knowledge, KIF1A has not yet been directly implicated in the pathogenesis of morphine tolerance. However, KIF1A carries AMPA receptors in rat neurons,[86] and chronic morphine in rats is associated with decreased surface expression of AMPA receptors in the NAc cells.[19] This accumbal glutamatergic alteration in the presence of up-regulated KIF1A may indicate an attempt by NAc neurons to compensate for decreased AMPA receptor expression, faulty transport of SVP by KIF1A or abnormal unloading of SVP from KIF1A. The up-regulation of KIF1A in the NAc can be explained by reduced anterograde KIF1A

transport from the NAc, unbalanced synthesis/degradation of KIF1A,[87] or a dysfunction of mechanisms inhibiting the peripheral buildup of KIF1A,[88] all of which may interfere with the axonal transport of SVP by KIF1A and may be associated with altered synaptic plasticity in the MDS.

4.3 Differentially expressed proteins: metabolism

In the present study, soluble epoxide hydrolase (SEH; +1.69), eukaryotic elongation factor 1 γ (eEF1γ; +1.25) and selenium-binding protein 1 (SBP1; +1.17) were identified as differentially expressed proteins linked to metabolism in the NAc associated with morphine tolerance.

SEH, also named cytosolic epoxide hydrolase or epoxide hydrolase 2, catalyzes the hydrolysis of epoxide, which are derived from metabolism of endogenous or xenobiotic substances via oxidation processes such as the cytochrome P450 monooxygenase system.[89] In the rodent brain, SEH is predominantly localized in neurons and abundantly expressed in the striatum.[90] SEH in the human brain is distributed predominantly in the oligodendrocytes and neuronal cell bodies.[91] SEH is responsible for the metabolism of epoxyeicosatrienoic acids (EETs) and leukotoxin. EETs in the rat brain are produced by astrocytes.[92] EETs are cytochrome P450 epoxygenases-derived metabolites of arachidonic acid and function as endogenous chemical mediators[93] with vasodilatory, anti-inflammatory,[94] antinociceptive[95] and anti-hyperalgesic[96] properties; besides, EETs are thought to be neuroprotective because they regulate cerebral blood flow[97] and protect against ischemic brain damage.[90] These beneficial effects of EETs are attenuated by metabolism by SEH.[93] On the other hand, the toxicity of leukotoxin such as depressed mitochondrial respiration depends on its hydrolysis by SEH to toxic diols.[94] Thus we postulate that up-regulated SEH in the NAc may lead to injury of cells therein and dysfunction of the MDS.

eEF1 is essential for GTP-dependent translational elongation in protein biosynthesis by transport of aminoacyl tRNA to ribosomes. It consists of eEF1A (a G-protein) and eEF1B (the guanine nucleotide exchange factor), the later composed of at least four subunits (α, β, γ and δ).[98,99] The exact functions of eEF1Bγ (or eEF1γ) remain largely speculative. It may act as a scaffold for the different subunits in the

eEF1B complex[99] and enhance the guanine nucleotide exchange activity of eEF1Bβ.[100] eEF1Bγ may interact with endoplasmic reticulum and cytoskeletal structures and thus anchor the multi-subunit eEF1B therein and regulate protein synthesis.[100-102]

The association between morphine tolerance and eEF1Bγ up-regulation, as revealed in the present study, has not been reported previously. eEF1Bβγ complex was found to co-localize and interact with dopamine D3 receptor (D3R) on the plasma membrane *in vitro*.[103] A high level of D3R mRNA is expressed in the NAc.[104] D3R mRNA is increased in dopaminergic and dopaminoceptive regions of the rat brain in response to chronic morphine.[105] D3R agonist 7-hydroxydipropylaminotetralin (7-OH-DPAT) attenuates the development of morphine tolerance in rats,[106] whereas a remarkable enhancement of morphine-induced rewarding effect is noted in D3R knock-out mice.[107] We postulate that eEF1Bγ may act either as a D3R antagonist to accentuate the development of morphine tolerance, or as a D3R agonist to normalize the dysregulated dopamine transmission associated with morphine tolerance.

Mammalian SBP1, a 56 kDa protein that binds selenium, was first found in mouse liver[108] and proposed to be a growth regulatory protein.[109] Reduced SBP1 expression has been reported in multiple human malignancies.[110-113] Neuronal and glial expression of SBP1 in the brain has been demonstrated,[114] but its role in the brain remains elusive. SBP1 is up-regulated in the brain of schizophrenic and psychotic patients.[114,115] SBP1 may take part in late stages of intra-Golgi protein transport and regulate vesicular docking/fusion.[116] Moreover, in human glioma and neuroblastoma cells, SBP showed polarized localization exclusively at the growing tips.[117] Therefore, SBP1 may have a role in rapid outgrowth of brain cells and thus take part in neuroplasticity associated with the development of morphine tolerance.

4.4 Differentially expressed proteins: mitochondrial

In the present study, antioxidant protein isoform 1 (AOP-1; –1.37) and ubiquinol-cytochrome c reductase (–1.19) were identified as differentially expressed mitochondrial proteins in the NAc related to morphine tolerance.

Mitochondrial AOP-1, or peroxiredoxin 3 (PRDX3) protein, belongs to a family of

thiol-based peroxidases that reduce peroxides and protect cells against oxidative damage.[118] PRDX3 in the brain is expressed in neurons.[119] Its *in vivo* functions are unclear. Inactivation of PRDX3 enhances apoptosis,[120] which may be related to the apoptotic effect of H_2O_2. PRDX3 also protects rat hippocampal neurons from excitotoxicity.[121] PRDX2 is down-regulated in the NAc of cocaine overdose victims; the phenomenon may represent the result of H_2O_2 accumulation and oxidative stress.[44] Likewise, down-regulation of PRDX3 in the NAc of morphine-tolerant pigs, as revealed in the present study, may predispose to oxidative damage in the NAc and dysfunction of the MDS.

Ubiquinol-cytochrome c reductase (electron transport complex III) is a mitochondrial oxidative phosphorylation enzyme. Down-regulation of this enzyme in the NAc of morphine-tolerant pigs was revealed in the present study. Decreased levels of the components of the ubiquinol-cytochrome c reductase complex were found in the brains of rats with morphine dependence[122] as well as in the frontal cortex of individuals with depression.[63] The significance of these findings awaits further investigation.

4.5 Differentially expressed proteins: miscellaneous

In the present study, hsp60 1 (chaperonin) isoform 1 (–1.322) and CK-B or CK (–1.31, –1.38, –1.36, –1.27) were identified as differentially expressed proteins in the NAc associated with morphine tolerance.

Hsp60, or chaperonin 60, is a molecular chaperone[123] that assists the ATP-dependent folding of nascent polypeptides into functional three-dimensional conformations, counteracts unwanted protein aggregation and protects proteins from denaturation following stress conditions (such as high temperature or ischemia).[124] Besides the preservation of the integrity of proteome, hsp60 is also responsible for facilitating the transport and maintenance of mitochondrial proteins.[125]

Hsp has been proposed to defend against neurotoxicity.[126,127] Antibodies to hsp60 have been connected to schizophrenia, which may be explained by inhibition of neuroprotection.[128,129] Induction of hsp60 in rodents with recombinant adenoviruses expressing hsp60 protects hippocampal neurons from ischemic damage.[130] Therefore,

down-regulation of hsp60 in the NAc of morphine-tolerant pigs, as revealed in the present study, may result in defect of some proteins (presumably from loss of "protein quality control"[131]) and thus impair the functions of the NAc neurons and contribute to the development of morphine tolerance.

In mammals, there are three dimeric isozymes (CK-BB, CK-MB and CK-MM) of CK in the cytoplasm of excitable cells and tissues with high and fluctuating energy consumption such as brain and muscle. All of the isozymes catalyze the reversible transfer of high-energy phosphate between ATP and creatine phosphate and thus act as a critical energy buffer and regulator in tissues with high energy demands.[132,133]

Normal CK activity is crucial to brain function.[134] Some evidence suggests an association between functional impairment of CK and neurological diseases, possibly mediated by compromised energy metabolism in the nervous system. Decreased level or activity of CK-B (brain-type CK) in the brain was reported in individuals with schizophrenia, Alzheimer's disease or Pick's disease.[135-138] As drug addiction is the results of neuroadaptation, it is reasonable to speculate that there are concomitant alterations of energy metabolism in the relevant brain nuclei. Opiates cause a global reduction in brain metabolism;[139] a decline in CK-B level was also observed in the NAc in rhesus monkeys self-injecting cocaine.[45] In contrast, the psychoactive component of marijuana caused up-regulated CK-B in normal human astrocytes.[140] The inconsistency might be explained by differences between drugs and/or species. In the present study, there were 4 adjacent protein spots (524, 527, 529 and 530) identified by MS as CK-B (or CK) with similar, significantly decreased level in the morphine group. These may represent different degrees of phosphorylation.

4.6 Conclusion

Morphine tolerance poses a severe problem to the patients in pain and clinicians, but its underlying neuroadaptive and biochemical changes are far from clear. In the present study we used proteomics approach to examine the NAc proteome in a pig model of morphine tolerance. The differentially expressed proteins thus identified are pertinent to cytoskeleton, cell signaling, metabolism, mitochondrial function and chaperonin. Some of the identified proteins are previously not known to be involved in

morphine tolerance. The findings of the present study proves the feasibility of a pig model in the field of neuroproteomics and shed light on the interplay of molecular and chemical mechanisms associated in the pathophysiology of morphine tolerance. The proteins identified in the present study may serve as targets for future studies on the diagnosis and treatment of opioid tolerance.

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