Phosphoproteomics and Bioinformatics Analyses of Spinal Cord Proteins in Rats with Morphine Tolerance

Wen-Jinn Liaw¹,²,³, Cheng-Ming Tsoa²,⁴, Go-Shine Huang², Chin-Chen Wu³,⁵, Shung-Tai Ho⁴, Jhi-Joung Wang⁶, Yuan-Xiang Tao⁷, Hao-Ai Shui⁸*

¹ Department of Anesthesiology, Tungs’ Taichung MetroHarbor Hospital, Taichung, Taiwan, ²Department of Anesthesiology, Tri-Service General Hospital, National Defense Medical Center, Taipei, Taiwan, ³ Department of Pharmacology, National Defense Medical Center, Taipei, Taiwan, ⁴Department of Anesthesiology, Taipei Veterans General Hospital and National Yang-Ming University, Taipei, Taiwan, ⁵Department of Pharmacology, College of Medicine, Taipei Medical University, Taipei, Taiwan, ⁶Departments of Anesthesiology, Medical Research, Chi Mei Medical Center, Tainan, Taiwan, ⁷Department of Anesthesiology, Rutgers, The State University of New Jersey, New Jersey Medical School, Newark, New Jersey, United States of America, ⁸Graduate Institute of Medical Sciences, National Defense Medical Center, Taipei, Taiwan

Abstract

Introduction: Morphine is the most effective pain-relieving drug, but it can cause unwanted side effects. Direct neuraxial administration of morphine to spinal cord not only can provide effective, reliable pain relief but also can prevent the development of supraspinal side effects. However, repeated neuraxial administration of morphine may still lead to morphine tolerance.

Methods: To better understand the mechanism that causes morphine tolerance, we induced tolerance in rats at the spinal cord level by giving them twice-daily injections of morphine (20 μg/10 μL) for 4 days. We confirmed tolerance by measuring paw withdrawal latencies and maximal possible analgesic effect of morphine on day 5. We then carried out phosphoproteomic analysis to investigate the global phosphorylation of spinal proteins associated with morphine tolerance. Finally, pull-down assays were used to identify phosphorylated types and sites of 14-3-3 proteins, and bioinformatics was applied to predict biological networks impacted by the morphine-regulated proteins.

Results: Our proteomics data showed that repeated morphine treatment altered phosphorylation of 10 proteins in the spinal cord. Pull-down assays identified 2 serine/threonine phosphorylated sites in 14-3-3 proteins. Bioinformatics further revealed that morphine impacted on cytoskeletal reorganization, neuroplasticity, protein folding and modulation, signal transduction and biomolecular metabolism.

Conclusions: Repeated morphine administration may affect multiple biological networks by altering protein phosphorylation. These data may provide insight into the mechanism that underlies the development of morphine tolerance.

Citation: Liaw W-J, Tsoa C-M, Huang G-S, Wu C-C, Ho S-T, et al. (2014) Phosphoproteomics and Bioinformatics Analyses of Spinal Cord Proteins in Rats with Morphine Tolerance. PLoS ONE 9(1): e83817. doi:10.1371/journal.pone.0083817

Introduction

Morphine is primarily used to treat severe pain caused by acute injuries and chronic diseases. However, systematic administration of morphine can cause many side effects, including impairment of mental and physical functions, psychological dependence, addiction, and tolerance [1,2]. Since most of the side effects of morphine occur in the supraspinal regions of the central nervous system (CNS), direct neuraxial administration of morphine to act on spinal cord can prevent the supraspinal side effects and provide effective pain relief [2]. However, repeated neuraxial administration of morphine can still lead to tolerance, which is characterized by loss of analgesic effect of the initial effective dose [3].
animal model of morphine tolerance would provide a better understanding of the mechanism of this illness [7].

The development of morphine tolerance is thought to be associated with dysregulated phosphorylation of proteins for two reasons. First, morphine exerts its pharmacologic effects by acting at opioid receptors [9], which transduce signals and modulate protein activity via protein phosphorylation. Disturbance of the pharmacologic effects and signal transduction of morphine have been suggested to cause side effects of morphine [9,10]. Second, studies of cultured cells and brain tissue have shown that morphine and other opioid agonists can affect phosphorylation of certain proteins [11,12]. We hypothesize that a specific set of phosphoproteins is likely to be involved in the pathogenesis of morphine tolerance. However, the set of phosphoproteins, i.e. the morphine tolerance-related phosphoproteome, has never been explored, especially in the spinal region.

Phosphoproteomics is used to study the phosphorylation of many proteins (the phosphoproteome), rather than individual proteins in a biological sample [13]. Bioinformatics uses computational algorithms to ascertain the physiologic impact of proteins at the systems level [14]. Both approaches can be used to identify proteins whose roles in a disorder have not been established by other traditional methods. To the best of our knowledge, however, these two approaches have never been used to explore the state of phosphoproteins and their biological networks in spinal cord as they relate to development of morphine tolerance. In this study, we used phosphoproteomics and bioinformatics analysis of spinal cord proteins in rats with morphine tolerance to evaluate the impact of morphine on spinal cord in terms of protein phosphorylation, and to better understand the pathophysiologic mechanism that underlies morphine tolerance.

Materials and Methods

Animals and drug treatment

All animal experiments were carried out with the approval of the Institutional Animal Care and Use Committee at the National Defense Medical Center, Taiwan, and were consistent with the ethical guidelines of the National Institutes of Health and the International Association for the Study of Pain.

Male Sprague-Dawley rats (250–300 g) were used in this study. As shown in Fig. 1A, we first implanted a polyethylene-10 catheter into the subarachnoid space of the rats at the rostral level of the spinal cord lumbar enlargement segments as described previously [4,5,15–17]. The animals were allowed to recover for a week; animals that developed neurologic deficits postoperatively were removed from the study. After recovering from the catheter implantation, the rats were injected intrathecally through the catheter twice daily with saline (10 g/10 mL; control group; n = 6) or morphine sulfate (20 mg/10 mL saline, Sigma, St. Louis, MO; n = 6) for 4 consecutive days [5,18]. On day 5, we injected rats in both groups with morphine sulfate (20 mg/10 mL) to evaluate the analgesic potency [3,19].

Nociception tests for morphine efficacy

Nociception tests were carried out at baseline (1 day before the first morphine or saline injection) and on day 5 (30 min after the final injection) as shown in Fig. 1A. Noxious radiant heat (Model 33B Analgesia Meter; IITC Life Science, Woodland Hills, CA, USA) was applied to the plantar surface of each hind paw of the animals. Paw withdrawal latency (PWL) of both hind paws was measured as the time between heat application and paw withdrawal. A cutoff time for the heat application was set to 20 s to prevent tissue damage to the paw [3].

Final and baseline PWLs were compared and used to calculate maximal possible analgesic effect (MPAE) of morphine. MPAE was calculated by the formula: [(final PWL – baseline PWL)/(cutoff time – baseline PWL)] x 100% [5].

Sample preparation and two-dimensional gel electrophoresis (2-DE)

After nociception testing, the animals were killed and lumbar enlargement segments of spinal cord were harvested. The spinal tissues were immediately lysed and homogenized with a sonication probe in 0.5 mL of lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, and 0.5% IPG buffer [pH 3–10]). The lysates were centrifuged at 15000 g for 15 min at room temperature to remove insoluble debris. Protein concentration in the supernatant was determined by a modified Bradford method [20].

The spinal proteins (250 μg) from control and morphine-treated groups were loaded onto IPG strips (Immobiline DryStrip 3–10, GE Healthcare, Piscataway, NJ, USA) for simultaneous rehydration. Isoelectric focusing was performed by using a voltage-time program of 50 V for 12 h, 500 V for 1 h, 1000 V for 1 h, and 7000 V to give a total of 140,000 V-h. Immediately after focusing, the IPG strips were equilibrated for 15 min in equilibration buffer (6 M urea, 2% sodium dodecyl sulfate [SDS]; 50 mM Tris–HCl, pH 8.4, and 30% glycerol) containing 1% dithiothreitol, and then for 15 min in equilibration buffer containing 2.5% iodoacetamide. The second dimension separation was carried out at 15°C with a vertical electrophoresis system (GE Healthcare) in 1 mm 12.5% acrylamide gels run at 20 mA/gel.

Gel staining and image analysis

After electrophoresis, the 2-DE gels were subjected to fluorescence staining with Pro-Q Diamond phosphoprotein dye (Invitrogen, Carlsbad, CA, USA) for detection of protein phosphorylation levels [21]. Briefly, the gels were fixed in fixation solution (50% methanol, 10% acetic acid), washed with distilled water twice, then stained by the Pro-Q Diamond dye for 4 hours. The gels were destained in three successive washes of destaining solution (20% acetonitrile, 50 mM sodium acetate [pH 4]) and three washes of distilled water. Images were obtained by scanning the 2-DE gels with a Typhoon Trio laser scanner (GE Healthcare). For measuring total protein levels, the stained gels were washed with methanol solution (50% methanol, 10% acetic acid), to remove Pro-Q Diamond dye, and were restained with SYPRO Ruby fluorescent dye according to the manufacturer’s protocol. Gel images were obtained again by scanning the 2-DE gels with a Typhoon Trio laser scanner.

Spot detection, gel matching, and spot quantification were carried out with 2-DE gel analysis software (ImageMaster 2D platinum, GE Healthcare). To correctly estimate phosphorylation levels, we normalized the phosphorylation intensities of an individual protein revealed by Pro-Q Diamond dye to the total expression level of the same protein revealed by SYPRO Ruby fluorescent dye [21]. The molecular weight (Mr) and isoelectric point (pI) of each protein spot were estimated by the software based on the positions of standard markers and standard pI positions, respectively.

In-gel digestion, matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectroscopy (MS) analysis, and protein identification

Proteins whose phosphorylation level differed significantly between control and morphine-treated groups were excised from the 2-DE gels and digested in the gel with trypsin. The digested
proteins were subjected to MALDI-TOF MS analysis (Autoflex II, Bruker Daltonics, Bremen, Germany) to obtain the peptide mass fingerprint (PMF). Each mass spectrum was obtained from the average of signals generated from at least 500 laser shots. The PMFs were processed by using Flexanalysis™ and Biotools™ software (Bruker Daltonics) and were used to search the UniProt database (http://www.uniprot.org/) by using the MS-Fit on-line search engine (http://prospector.ucsf.edu/). For each PMF search to identify a protein, the mass tolerance was set at 150 ppm, and one missed tryptic cleavage was allowed.

**Pull-down assays of phosphopeptide and phosphoprotein**

To identify the types and sites of phosphorylation in the most significantly changed protein (i.e. 14-3-3 proteins), we performed pull-down assays at both peptide and protein levels. For pull-down assay of a phosphopeptide, the selected protein was in-gel digested into many peptides, and phosphopeptides of the digested protein were precipitated by using Phos-trap™ magnetic beads (Perkin Elmer, San Jose, CA, USA) according to the manufacturer’s protocol [22]. MALDI-TOF MS was then used to reveal the precipitated phosphopeptides as well as the original total peptides. Precipitated proteins were then subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. Western blotting was carried out sequentially by electroblotting proteins onto polyvinylidene difluoride membranes, incubating the membranes with a mouse monoclonal anti-14-3-3 antibody (Chemicon International), and probing with anti-mouse HRP-conjugated secondary antibody. Bands were visualized with an ECL detection kit (Millipore, Billerica, MA, USA). The lysates also were subjected to SDS-PAGE and Western blotting to check the total amount of 14-3-3 protein and glyceraldehyde 3-phosphate dehydrogenase, which served as input controls.

**Bioinformatics analysis**

To further estimate the impact of morphine on biological networks, the bioinformatics tool STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) was used to elucidate biological networks regulated by the proteomics-identified phosphoproteins [23–25]. The networks were generated by algorithmically assembling the identified phosphoproteins and their interacting proteins from the STRING database. The biological networks were then classified into clusters by protein function [23–25].

**Statistics**

Student’s t-test was used for the statistical comparison of data from control and morphine-treated groups. Differences were considered significant at \( p < 0.05 \). The data are presented as the mean ± SD.
Results

Establishment of morphine tolerance in rats

PWLs and MPAEs were measured to ensure the development of morphine tolerance in rats after repeated injections of morphine. As shown in Fig. 1B, the baseline PWLs were similar between the control and morphine-treated rats ($p > 0.05$), whereas the final PWLs on day 5 were significantly shortened in morphine-treated rats as compared to those in control rats ($p < 0.05$). Because PWL inversely correlates with the extent of pain sensation, the shortened PWLs indicate a reduced analgesic effect of morphine. To ensure the development of morphine tolerance, we also quantified the degree of morphine antinociception by calculating MPAEs. As shown in Fig. 1C, MPAE was significantly less in the morphine-treated rats than in the control rats in both left and right paws ($p < 0.05$). Again, this quantitative data confirmed that the analgesic effect of morphine was reduced after repeated morphine injections and verified morphine tolerance.

Phosphoproteome profiles of spinal cord from control and morphine-treated rats

Comparative 2-DE-based proteomic analysis revealed a marked difference in phosphorylation pattern of spinal cord proteins between control and morphine-treated rats. Patterns of protein phosphorylation are shown by the representative 2-DE gels stained with ProQ Diamond phosphorylation detection dye. The same pair of 2-DE gels from panel A were restained with SyproRuby fluorescent dye to show the total protein expression profiles. The protein phosphorylation intensities of the upper gels in panel A were normalized to total protein expression intensities obtained from the lower gels in panel B. The proteins that showed a significant difference in the normalized phosphorylation level are indicated by arrows and labeled with the same numbers used in Table 1. The molecular mass is indicated on the right, and the pI range is shown at the bottom of each gel.

doi:10.1371/journal.pone.0083817.g002

Figure 2. Representative 2-DE gel maps of phosphorylated proteins and total proteins from spinal cord of control and morphine-treated rats. (A) Typical 2-DE gels representing phosphorylated proteins from control (left) and morphine-treated (right) rats; gels were stained with a ProQ Diamond phosphorylation detection dye. (B) The same pair of 2-DE gels from panel A were restained with SyproRuby fluorescent dye to show the total protein expression profiles. The protein phosphorylation intensities of the upper gels in panel A were normalized to total protein expression intensities obtained from the lower gels in panel B. The proteins that showed a significant difference in the normalized phosphorylation level are indicated by arrows and labeled with the same numbers used in Table 1. The molecular mass is indicated on the right, and the pI range is shown at the bottom of each gel.

Quantification and identification of differentially phosphorylated proteins

The magnified images in Fig. 3A show phosphorylation level (P) and total amount (T) of individual protein spots in control and morphine-treated groups; intensities of protein phosphorylation were divided by corresponding intensities of total protein to give the normalized phosphorylation levels (P/T ratios) shown in Fig. 3B [21]. Differentially phosphorylated proteins were identified by PMF with MALDI-TOF MS, and their various characteristics are shown in Table 1. The proteins included glial fibrillary acidic protein (Gap), alpha-internexin (Ina), heat shock 70 kDa protein 5 (Hspa5), 14-3-3 protein gamma (Ywhag), 14-3-3 protein zeta/delta (Ywhaz), prohibitin (Phb), tyrosyl-tRNAsynthetase (Yars), gamma-enolase (Eno2), fructose-bisphosphatealdolase C (Aldoc).
and collectin sub-family member 10 (Colec10). Phosphorylation levels of Gfap, Ina, Phb, and Colec10 were decreased in the morphine-treated group, whereas phosphorylation levels of the other six proteins were increased.

**Identification of phosphoproteins involved in neuroplasticity.** Gfap (Fig. 2, spot 1; Table 1), a glia cell-specific cytoskeleton protein [26], and Ina (Fig. 2, spot 2, Table 1), a neuron-specific cytoskeleton protein [27], were hypophosphorylated after morphine treatment. Both cytoskeletal proteins play roles in neuroplasticity.

**Identification of chaperones.** Chaperones are involved in maintaining proper conformations of and modulating activities of other proteins. After repeated injection of morphine, phosphorylation levels of one endoplasmic reticulum (ER)-specific chaperone (Hspa5; Fig. 2, spot 3; Table 1) [28] and two 14-3-3 proteins (Fig. 2, spots 4 and 5; Table 1) [29] were increased.

**Identification of signaling scaffold protein.** Scaffold proteins are key regulators of many signaling pathways. Repeated treatment with morphine reduced the phosphorylation level of Phb (Fig. 2, spot 6; Table 1), a scaffold protein that controls the signal transduction of PI3K/Akt, TGF-beta, and Ras/MAPK/ERK [30].

**Identification of enzymes involved in biomolecular metabolism.** The phosphorylation levels of three metabolic enzymes were increased in the morphine-treated group.
Table 1. Identified proteins.

| Spot No. | MOWSE score | Accession No. | Protein name abbreviation | Protein name | Theoretical Mr (kDa)/pI | Sequence coverage (%) |
|----------|-------------|---------------|---------------------------|--------------|-------------------------|-----------------------|
| 1        | 163000000   | P47819        | Gfap                      | Glial fibrillary acidic protein | 49.957/5.4            | 58.8                  |
| 2        | 312000000   | P23565        | Ina                       | Alpha-internexin                  | 56.116/5.2            | 58.8                  |
| 3        | 5574        | P06761        | Hspa5                     | Heat shock 70 kDa protein 5       | 72.348/5.1            | 43.1                  |
| 4        | 9645        | P61983        | Ywhag                     | 14-3-3 protein gamma               | 28.303/4.8            | 41.7                  |
| 5        | 139899      | P63102        | Ywhaz                     | 14-3-3 protein zeta/delta          | 27.771/4.7            | 51.4                  |
| 6        | 39333       | P67779        | Phb                       | Prohibitin                            | 29.82/5.6            | 37.5                  |
| 7        | 204         | Q4KM49        | Yars                      | Tyrosyl-tRNA synthetase              | 59.116/6.6            | 20.1                  |
| 8        | 16777       | P07323        | Eno2                      | Gamma-enolase                        | 47.141/5.0            | 27.9                  |
| 9        | 1253        | P09117        | Aldoc                     | Fructose-bisphosphate aldolase C     | 39.284/6.7            | 40.5                  |
| 10       | 45.3        | D4A7F6        | Colec10                   | Collectin sub-family member 10      | 30.628/7.5            | 11.2                  |

The spot number refers to the numbers in Fig. 2. The MOWSE (MOlecular Weight SEarch) score is used to identify proteins from the molecular weight of peptides produced by proteolytic digestion. Mr, theoretical molecular mass; pI, isoelectric point. The sequence coverage of matching peptides was calculated by using Biotools™ software.

doi:10.1371/journal.pone.0083817.t001

Figure 4. Phosphorylation of 14-3-3 proteins as shown by pull-down assays of phosphopeptide and phosphoprotein. (A) MALDI-TOF MS spectrum of total peptides derived from tryptic digestion of 14-3-3 protein Ywhaz (top panel), and a spectrum of phosphopeptides precipitated from total peptides of the digested 14-3-3 protein (bottom panel). The phosphorylated residues represented by the peaks were predicted to be serine and threonine. (B) Upper panel: Western blot showing that more 14-3-3 protein was precipitated by anti-phospho-serine/anti-phospho-threonine antibodies in morphine-treated spinal cord than in control spinal cord. Middle panel: Western blots showing that total 14-3-3 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) proteins in the lysates of spinal cord were similar in control and morphine-treated rats. Lower panel: Statistical and quantification data of phosphorylation of 14-3-3 proteins.

doi:10.1371/journal.pone.0083817.g004
enzymes were altered, including two glycolysis enzymes, Eno2 (Fig. 2, spot 8; Table 1) and Aldoc (Fig. 2, spot 9; Table 1) [31], and one protein synthesis-related enzyme Yars (Fig. 2, spot 7; Table 1).

Identification of types and sites of phosphorylation using pull-down assays

Pull-down assays were performed to identify the types and sites of phosphorylation in the most significantly changed protein (i.e. 14-3-3 proteins) in 2-DE. Phosphopeptide-enriching magnetic beads induced the precipitation of two major phosphopeptides (P1 and P2 in Fig. 4) from the total peptides of the in-gel digested 14-3-3 protein Ywhaz (spot 5). Based on their amino acid sequences, these peptides were predicted to be serine- and threonine-phosphorylated (Fig. 4A).

Immunoprecipitation of the phosphorylated proteins from total lysates of spinal cord samples and detection by Western blot analysis revealed that 14-3-3 proteins were more abundant in spinal cords from morphine-treated rats than in those from control rats, and confirmed the prediction that 14-3-3 proteins were serine- and threonine-phosphorylated (Fig. 4B).

Biological networks regulated by the morphine-affected phosphoproteins

Bioinformatics analysis was used to estimate the biological networks impacted by the 10 proteomics-identified phosphoproteins. The biological network clusters, shown in Fig. 5, consisted of cytoskeleton reorganization and neuroplasticity (cluster I), protein folding (cluster II), protein modulation (cluster III), signal transduction (cluster IV), and biomolecular metabolism (cluster V).

Discussion

To date, phosphoproteomics has not been used to analyze phosphorylation of spinal cord proteins after development of morphine tolerance. Our proteomics and bioinformatics data revealed that repeated intrathecal morphine injections dysregu-
lated the phosphorylation of 10 proteins in rat spinal cord, impacting biological networks associated with various physiologic functions of the CNS. Our results are novel in that the identified phosphoproteins have not been reported previously to be associated with morphine tolerance.

The nervous system requires a degree of plasticity to respond to external stimuli and insults. This plasticity is achieved through cytoskeletal reorganization, which is controlled by phosphorylation of cytoskeletal proteins [32–34]. Our study showed that phosphorylation states of two cytoskeletal proteins in the spinal cord, i.e. Gfap and Ina, were altered by repeated administration of morphine. GFAP is an Intermediate filament protein specifically expressed in astrocytes in the CNS, and it serves as a sensitive and specific indicator of CNS plasticity in neurotoxic conditions [35,36]. Ina is another intermediate filament protein expressed only in neurons, and can cause CNS plasticity by facilitating axonal neurite elongation [37]. These data are consistent with previous reports showing that morphine administration impact GFAP expression in certain brain areas [38], and support previously published evidence showing that morphine tolerance is actually a disorder of neuroplasticity [39,40].

We also identified several chaperone proteins in this study. Chaperones play a key role in preventing their target proteins from misfolding and aggregating into nonfunctional structures; hence, they ensure proper activity of the target proteins [41]. The phosphorylation level of an ER-specific chaperone, Hspa5 (also called GRP78), was significantly altered in the spinal cord after development of morphine tolerance [42,43]. Hspa5 is a major ER chaperone controlling the protein quality in the ER and protecting cells from ER stress, which is characterized by accumulation of misfolded proteins in ER [42,43]. Various stresses can upregulate the expression of Hspa5, and the overexpressed Hspa5 within and outside the ER to play a critical role in cell viability [44–46]. For example, Hspa5 protects neurons and astrocytes against mitochondria dysfunction and stress-induced apoptosis [44,46]. The effect of morphine on Hspa5 is consistent with previous reports demonstrating that another ER chaperone, BiP, also plays a pathophysiological role in the development of morphine tolerance [47].

Two of the identified chaperones belong to the 14-3-3 protein family [48,49]. 14-3-3 proteins are able to bind to the phosphorylated motifs of their partner proteins to affect protein activities [29]. Through functional modulation of the binding partners, 14-3-3 proteins are involved in multiple processes running in the cell, including metabolism, apoptosis, cell cycle and gene transcription [50,51]. It has been known that posttranslational modifications playing important roles in regulation of 14-3-3 activity, and this regulation plays an important role in cytoskeletal reorganization of intermediate filaments in neurons [52]. Especially, 14-3-3 proteins modulate the sensitivity and trafficking of opioid and NMDA receptors [53,54], both of which are known to be involved in the development of morphine tolerance [39]. Thus, the dysregulated phosphorylation of 14-3-3 proteins seen in this study might contribute to the pathophysiology of morphine tolerance through opioid and/or NMDA receptors.

Like chaperones, scaffold proteins are a type of regulatory protein that modulates cellular processes through interaction with multiple partner proteins. In our study, we identified Phb, a scaffold protein originally found in mitochondria [55]. Within mitochondria, Phb is able to maintain mitochondrial integrity and suppress free radical production [36]. However, Phb also has functions outside of mitochondria, such as controlling proliferation, apoptosis, and transcription [57]. In addition, Phb is neuroprotective against stress-induced neuronal death [36], and plays a role in modulating PI3K/Akt and Ras/MAPK/ERK signal transduction [30]. The altered phosphorylation state of Phb in morphine-treated rats suggests that morphine influences some of the known functions of Phb in the spinal cord.

In conclusion, our proteomics data showed that repeated intrathecal injection of morphine dysregulated the phosphorylation of 10 proteins in the spinal cord. Bioinformatics analysis revealed five functional networks of proteins that are affected and known to be involved in cytoskeletal reorganization, neuroplasticity, protein folding and modulation, signal transduction, and biomolecular metabolism (Fig. 5A). These proteins are known to be expressed in different subcellular organelles, associating with neuroplasticity, ER stress, mitochondrial dysfunction, receptor trafficking and neuroprotection (Fig. 5B). Our data may shed light on the multiple phosphoproteome mechanisms that underlie the development of morphine tolerance.

Author Contributions
Conceived and designed the experiments: WJL HAS. Performed the experiments: WJL HAS. Analyzed the data: CCW. Contributed reagents/ materials/analysis tools: STH JWW YJT. Wrote the paper: CMT WJL GSH YJT HAS.

References
1. Cherny N, Ripamonti C, Pereira J, Davis C, Fallon M, et al. (2001) Strategies to manage the adverse effects of oral morphine: an evidence-based report. J Clin Oncol 19: 2542–2554.
2. Gerber HR (2005) Intrathecal morphine for chronic benign pain. Best Pract Res Clin Anaesthesiol 17: 429–442. 3. Olsen RK, Harvey S (2001) Neuraxial infusion in patients with chronic intractable cancer and noncancer pain. Curr Pain Headache Rep 5: 241–249.
4. Shui HA, Ho ST, Wang JJ, Wu CC, Lin CH, et al. (2007) Proteomic analysis of spinal protein expression in rats exposed to repeated intrathecal morphine injection. Proteomics 7: 796–803.
5. Liaw WJ, Zhang B, Tao F, Yaster M, Johns RA, et al. (2004) Knockdown of spinal cord post synaptic density protein-95 prevents the development of morphine tolerance in rats. Neuroscience 123: 11–15.
6. Gustein HB, Trujillo KA (1993) MK-801 inhibits the development of morphine tolerance at spinal sites. Brain Res 626: 332–334.
7. Menard DP, van Rossum D, Kar S, Jolicour FB, Jhamandas K, et al. (1995) Tolerance to the antinociceptive properties of morphine in the rat spinal cord: alteration of calcium- and peptide-like immunostaining and receptor binding sites. J Pharmacol Exp Ther 273: 807–804.
8. Hauser KE, Mangoura D (1998) Diversity of the endogenous opioid system in development. Novel signal transduction translates multiple extracellular signals into neural cell growth and differentiation. Perspect Dev Neurobiol 5: 437–449.
9. Berhow MT, Hira N, Neider EJ (1996) Regulation of ERK (extracellular signal regulated kinase), part of the neurotrophin signal transduction cascade, in the rat mesolimbic dopamine system by chronic exposure to morphine or cocaine. J Neurosci 16: 4707–4715.
10. Ohnaka M, Terunuma L (1989) Prolonged exposure of human neuroblastoma SH-SY5Y cell line to morphine and oxotremorine modulates signal transduction in adenylyl cyclase system. Brain Res 475: 291–296.
31. Ramagli L (1999) Quantifying protein in 2-D PAGE solubilization buffer. In: Altschuld KE, editor. 2-D Proteome analysis protocols. Totowa, NJ: Humana Press. pp. 99-103.
32. Chen CC, Shui HA, Wu CH, Wang CY, Sun GH, et al. (2009) Motility and protein phosphorylation in healthy and asthenozoospermic sperm. J Proteome Res 8: 5382-5386.
33. Larsson MR, Thimgneh TE, Jensen ON, Roepstorff P, Jorgensen T (2005) Highly selective enrichment of phosphorylated peptides from peptide mixtures using titanium dioxide microcolumns. Mol Cell Proteomics 4: 873-886.
34. Jensen LJ, Kuhn M, Stark M, Chaffon S, Grevey C, et al. (2009) STRING 8—a global view on proteins and their functional interactions in 630 organisms. Nucleic Acids Res 37: D414-D418.
35. Shui HA, Hsia CW, Chen HM, Chang TC, Wang CY (2012) Proteomics and bioinformatics analysis of lovastatin-induced differentiation in ARO cells. J Proteomics 75: 1170-1180.
36. Chai NC, Shui HA, Wu CH, Wang CY, Sun GH, et al. (2011) Impact of taxol on dermal papilla cells—a proteomics and bioinformatics analysis. J Proteomics 74: 2760-2773.
37. Rodnight NR, Goncalves CA, Wolchuk ST, Leal R (1997) Control of the phosphorylation of the astrocyte marker glial fibrillary acidic protein (GFAP) in the immature rat hippocampus by glutamate and calcium ions: possible key factor in astrocyte plasticity. Braz J Med Biol Res 30: 325-338.
38. Yuan A, Rao MV, Sasaki T, Chen Y, Kumar A, et al. (2006) Alpha-internexin is structurally and functionally associated with the neurofilament triplet proteins in the mature CNS. J Neurosci 26: 10006-10019.
39. Guzel E, Basar M, Ocak N, Aricil A, Kavvisi UA (2011) Bidirectional interaction between unfolded-protein-response-key protein HSPA5 and estrogen signaling in human endometriosis. J Biolog Reprod 85: 121-127.
40. Yaffe MB (2002) How do 14-3-3 proteins work?—Gatekeeper phosphorylation and the molecular arrest hypothesis. FEBS Lett 531: 53-57.
41. Mishra S, Andre SR, Nyomba BL (2010) The role of prohibitin in cell signaling. FEBS J 277: 3937-3946.
42. Hafner A, Obermaier N, Kos J (2012) gamma-Enolase C-terminal peptide promotes cell survival and neurite outgrowth by activation of the PI3K/Akt and MAPK/ERK signalling pathways. Biochem J 434: 439-450.
43. Rex CS, Gavin CF, Rubio MD, Kramar EA, Chen LY, et al. (2010) Myosin IIb regulates actin dynamics during synaptic plasticity and memory formation. Neuron 67: 603-617.
44. Sekino Y, Kojima N, Shirao T (2007) Role of actin cytoskeleton in dendritic spine morphogenesis. Neurochem Int 51: 92-104.
45. Ferrer-Alon M, Garcia-Sevilla JA, Jaquet PE, La Harpe R, Riederer BM, et al. (2000) Regulation of nonphosphorylated and phosphorylated forms of neurofilament proteins in the prefrontal cortex of human opioid addicts. J Neurosci Res 61: 330-349.
46. O’Callaghan JP, Sirum K (2005) Glial fibrillary acidic protein and related glial proteins as biomarkers of neurotoxicity. Expert Opin Drug Saf 4: 433-442.
47. Lewis GP, Fisher SK (2003) Up-regulation of glial fibrillary acidic protein in response to retinal injury: potential role in glial remodeling and a comparison to vimentin expression. Int Rev Cytol 230: 263-290.
48. Wang M, Ye R, Barron E, Baumeister P, Mao C, et al. (2010) Essential role of the unfolded protein response regulator GRP78/BiP in protection from neuronal apoptosis. Cell Death Differ 17: 483-491.
49. Doi T, Tanabe S, Jin H, Mimura N, Yamamoto T, et al. (2010) BiP, an endoplasmic reticulum chaperone, modulates the development of morphine antinociceptive tolerance. J Cell Mol Med 14: 2816-2826.
50. Exe K, Moroni M, D’Accielli F, Houllihan LM, Lakas RJ, et al. (2006) Chaperone protein 14-3-3 and protein kinase A increase the relative abundance of low agonist sensitivity human alpha 4 beta 2 nicotinic acetylcholine receptors in Xenopus oocytes. J Neurochem 90: 867-885.
51. Foucault I, Liu YC, Bernard A, Deckert M (2003) The chaperone protein 14-3-3 interacts with 3BP2/SH3BP2 and regulates its adapter function. J Biol Chem 278: 7146-7153.
52. Obalova V, Silhan J, Boura E, Teisinger J, Obal T (2008) 14-3-3 proteins: a family of versatile molecular regulators. Physiol Res 57 Suppl. 3: S11-21.
53. Hermetking H, Benzinger A (2006) 14-3-3 proteins in cell cycle regulation. Semin Cancer Biol 16: 103-192.
54. Shchukin NN, Gussev NB (2010) 14-3-3 proteins and regulation of cytoskeleton. Biochemistry (Mosc) 75: 1528-1546.
55. Chen RS, Roche KW (2009) Growth factor-dependent trafficking of cerebellar NMDA receptors via protein kinase B/Akt phosphorylation of NR2C. Neuron 62: 471-478.
56. Li JG, Chen C, Huang P, Wang Y, Liu-Chen LY (2010) Essential roles for cell proliferation and cristae morphogenesis. Biochim Biophys Acta 1793: 27-32.
57. Zhao P, Qian L, D’Aurelio M, Cho S, Wang G, et al. (2012) Prohibitin reduces mitochondrial free radical production and protects brain cells from different injury modalities. J Neurosci 32: 583-592.
58. Thieris AL, Sitarasan SV (2011) The role and therapeutic potential of prohibitin in disease. Biochim Biophys Acta 1815: 1137-1143.
59. Sharma SK, Yashpal K, Fundytus ME, Sauriol F, Henry JL, et al. (2003) The mechanisms of regulation of glycolytic enzymes in rat liver under morphine intoxication]. Biomed Khim 50: 204-209.
60. Lelevich SV (2007) [Mechanisms of regulation of glycolytic enzymes in rat liver under morphine intoxication]. Biomed Khim 53: 109-123.
61. Ouyang YB, Xu LJ, Emery JF, Lee AS, Giffard RG (2011) Overexpressing GRP78 influences Ca2+ handling and function of mitochondria in astrocytes after ischemia-like stress. Mol Biotechnol 48: 279-290.
62. Sun S, Wang M, Lee AS, Thompson RF (2011) Impaired eukaryon cell division in the absence of MnCEN proteins. J Neurosci 33: 2678-2688.
63. Yonemura Y, Larkin D, Lara-Lemus R, Ramos-Castaneda J, Liu M, et al. (2008) Endoplasmic reticulum (ER) chaperone regulation and survival of cells compensating for deficiencies in the ER stress response, kinase, PERK. J Biol Chem 283: 17102-17109.
64. Quinones OJ, de Ridford GG, Pizzo SV (2008) GRP78: a chaperone with diverse roles beyond the endoplasmic reticulum. Histochemistry 23: 1499-1511.
65. Ouyang YB, Xu LJ, Emery JF, Lee AS, Giffard RG (2011) Overexpressing GRP78 influences Ca2+ handling and function of mitochondria in astrocytes after ischemia-like stress. Mol Biotechnol 48: 279-290.