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Quantitative analysis of brain nuclear phosphoproteins identifies developmentally regulated phosphorylation events

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Abstract

Protein phosphorylation is a globally adopted and tightly controlled post-translational modification, and represents one of the most important molecular switching mechanisms that govern the entire spectrum of biological processes. In the central nervous system, it has been demonstrated that phosphorylation of key proteins mediating chromatin remodeling and gene transcription plays an important role controlling brain development, synaptogenesis, learning and memory. Many studies have focused on large scale identification of phosphopeptides in brain tissue. These studies have identified phosphorylation site specific motifs useful for predicting protein kinases substrates. In this study, we applied a previously developed quantitative approach, stable isotope labeling of amino acids in mammals (SILAM), to quantify changes in the phosphorylation of nuclear proteins between a postnatal day one (p1) and a p45 rat brain cortex. Using a ¹⁵N labeled rat brain as an internal standard, we quantified 705 phosphopeptides in the p1 cortex and 1477 phosphopeptides in the p45 cortex, which translates to 380 and 585 phosphoproteins in p1 and p45 cortex, respectively. Bioinformatic analysis of the differentially modified phosphoproteins revealed that phosphorylation is upregulated on multiple components of chromatin remodeling complexes in the p1 cortex. Taken together, we demonstrated for the first time the usefulness of employing stable isotope labeled rat tissue for global quantitative phosphorylation analysis.

Keywords

phosphorylation; quantification; mass spectrometry; brain development; nucleus

Introduction

The covalent addition of a phosphate group onto a serine, threonine, or tyrosine residue is predicted to occur on almost a third of proteins in higher organisms ¹. In fact, protein phosphorylation by kinases and dephosphorylation by phosphatases is by far the most effective and versatile molecular switching mechanism, which governs the entire spectrum of biological processes, from the flow of genetic information to the control of cell cycle, cell growth, differentiation and death. In the nucleus, it has been previously demonstrated that protein phosphorylation is an imperative regulatory mechanism controlling gene expression ^{2, 3}. In the brain, these phosphorylation dependent changes in gene expression have been demonstrated to regulate neuronal differentiation ⁴, synaptogenesis ⁵, as well as learning and memory ⁶. Mitogen-and stress-activated kinase 1 (MSK1) is a nuclear kinase with such a role. MSK1 knock-out mice exhibit a deficiency in histone phosphorylation in the brain. Phenotypically,

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these mice show deficits in multiple learning and memory tasks⁷, which is consistent with the general hypothesis that chromatin remodeling is necessary for memory formation⁸. The importance of protein phosphorylation is further emphasized by reports of its dysregulation in human diseases. For example, the tau protein promotes the assembly and maintenance of the microtubule structure, and these functions are regulated by phosphorylation⁹. Hyperphosphorylation of tau causes it to aggregate into neurofibrillary tangles, a histopathological hallmark of Alzheimer's disease and a hypothesized contributor to human dementia¹⁰. Furthermore, it has been suggested that the distinct combinations or arrays of phosphorylated and dephosphorylated proteins generate neuronal activity and modulate synaptic strength, but these phosphoprotein modules are poorly understood¹¹.

The study of phosphorylation on individual proteins has provided tremendous insight into individual protein function, however, these studies provide little information on phosphorylation modules. It is therefore difficult to appreciate the phosphorylation-mediated signal transduction cascade as a whole. In order to identify and understand these phosphorylation modules, large-scale mass spectrometry (MS) analysis has been employed¹². Mass spectrometry-based analyses of protein phosphorylation are hindered by two factors. First, phosphorylation is often present at a low stoichiometry making most phosphorylation difficult to detect in complex samples. To overcome this problem, enrichment techniques have been developed including immobilized affinity chromatography (IMAC)¹³⁻¹⁵, metal oxide affinity chromatography (MOAC)^{16, 17}, strong cation exchange chromatography¹⁸, and immunopurification¹⁹. Further hindering phosphoprotein analysis is the less informative fragmentation pattern generated by phosphopeptides compared to unmodified peptides. In the mass spectrometer, the phosphorylation modification is more labile than the peptide backbone during collision induced dissociation (CID), which can result in the neutral loss of phosphoric acid²⁰. This hampers the identification of the phosphopeptide, since this neutral loss fragmentation pattern yields fewer sequence ions. Precursor ion mapping and MS³ strategies, however, have taken advantage of this phenomenon to further improve the identification of phosphopeptides^{18, 21}. In the MS³ strategy, the neutral loss ion is selected for further fragmentation, and a more informative fragmentation pattern is produced allowing higher quality identification of the phosphopeptides¹⁸. Overall, hundreds to thousands of phosphoproteins have been identified from different biological samples using these methods^{15, 18, 22}.

To provide insight into the dynamics of these phosphorylation modules, laboratories have begun to quantify changes in phosphorylation events induced by different biological conditions. Quantification can be achieved by comparing a peptide with an identical peptide that is labeled with heavy isotopes (e.g. ¹³C or ¹⁵N)^{23, 24}. Given that a mass spectrometer can recognize the mass difference between light and heavy peptides, an abundance ratio between the labeled and unlabeled peptides can then be calculated from the respective ion chromatograms^{25, 26}. To label a protein sample with stable isotopes, either metabolic labeling or *in vitro* labeling can be employed^{27, 28}. Alterations in protein expression induced by a stimulus can be determined by analyzing two samples utilizing the same labeled internal standard²⁵. Metabolic labeling has advantages over *in vitro* labeling techniques since it exploits the cell's translational machinery to label all the proteins, while some *in vitro* labeling techniques use chemical reactions to label proteins with only certain functional groups²⁹. In addition, some *in vitro* techniques label peptides after digestion, and then the light and heavy samples are mixed, while metabolic labeling allows for the mixture of light and heavy samples before any sample preparation, such as the isolation of a specific organelle. Thus, metabolic labeling reduces the systematic errors that may accumulate during sample preparation between the heavy and light samples²⁵. Metabolic labeling is routinely used in simple systems, such as yeast and cultured mammalian cells, and has even been applied to simple organisms, including *C. elegans* and *D. melanogaster*, to quantify hundreds to thousands of unmodified

and phosphorylated peptides^{14, 30}. In comparison, few studies have performed large-scale quantitative phosphorylation analysis on mammalian tissue, and those that have employed *in vitro* labeling techniques^{31, 32}.

In order to study animal models of disease, we developed the strategy SILAM (Stable Isotope Labeling of Mammals) to metabolically label an entire mammal for quantitative MS analysis^{33, 34}. This strategy combines the necessity of studying mammalian tissues with the quantitative advantage of metabolic labeling. We previously demonstrated that labeling a rat with ¹⁵N for two generations had no adverse health effects and generated an entire animal highly enriched with ¹⁵N that was phenotypically normal³⁴. We validated the SILAM strategy by quantifying alterations in unmodified peptides in liver tissue induced by a systemic injection of cyclohexamide and in brain tissue during postnatal development^{33, 35}. Here, we demonstrate the use of SILAM for quantitative MS analysis of phosphorylation in mammalian brain during development.

Experimental Section

Nuclear enriched sample preparation

Sprague-Dawley rats were labeled with ¹⁵N as previously described^{33, 34}. Briefly, a female rat was fed a ¹⁵N labeled protein diet starting after weaning, remaining on the ¹⁵N protein diet throughout its pregnancy, and while feeding its pups. On postnatal day 45 (p45), the pups were subjected to halothane by inhalation until unresponsive, and the brains were quickly removed, frozen with liquid nitrogen, and stored at -80°C. The ¹⁵N enrichment was determined to be 94% using a previously described protocol³⁶. Brains from unlabeled Sprague-Dawley rats at p1 and p45 were obtained and stored in an identical manner as the ¹⁵N labeled brains except they were quickly dissected before freezing. All methods involving animals were approved by the Institutional Animal Research Committee and accredited by the American Association for Accreditation of Laboratory Animal Care.

Three snap-frozen p1 and p45 rat brain cortices, as well as ¹⁵N enriched rat brain were homogenized in the buffer (1 g of tissue/10 ml of buffer) containing 4mM HEPES, 0.32 M sucrose, protease and phosphatase inhibitors (Roche, Indianapolis, IN) in a Teflon hand held dounce grinder. After determining the protein concentration with a BCA protein assay (Pierce, Rockford, IL), homogenates from either p1 or p45 cortices were mixed at a 1:1 (wt/wt) ratio with the ¹⁵N brain homogenate. The ¹⁴N/¹⁵N mixture was added to 10ml of buffer and then, was centrifuged at 800 × g for 15 minutes. The resulting pellets were resuspended in 1ml of buffer containing 0.5% NP-40, and then, incubated on ice for 2 hours. The lysate was added to 10ml of buffer and centrifuged at 800 × g for 15 minutes. The resulting 0.5% NP-40 insoluble pellets were nuclear fraction³⁷ and were homogenized in 500ul of buffer and protein concentration was determined with a BCA protein assay.

Western Analysis

Nuclear preparations were solubilized in 2% SDS, separated by 8-16 % Tris-Glycine gel (Invitrogen, Carlsbad, CA), and electrophoretically transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA). Membranes were probed with the following antibodies: rabbit glutamate receptor 2/3 antibody and H3 histone antibody (Upstate Biotechnology, Temecula, CA). The membranes were then incubated with peroxidase-conjugated AffiPure goat anti-mouse or anti-rabbit secondary antibodies (Jackson ImmunoResearch, West Grove, PA), and the immunoreactive bands were visualized using SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL).

Trypsin digestion and enrichment of phosphopeptides using Immobilized Metal Affinity Chromatography (IMAC)

One milligram of the p1 and the p45 $^{14}\text{N}/^{15}\text{N}$ mixtures were precipitated with trichloroacetic acid at a final concentration of 20% for 30 minutes, and washed twice with cold acetone. The pellets were then solubilized by sonication with 4 M urea/5x Invitrosol (Invitrogen, Calsbad, CA), reduced with 10 mM dithiothreitol and alkylated with 10 mM iodoacetamide, both for 30 minutes. The solutions were diluted with 4x volumes of 100 mM Tris-HCl, and then digested with trypsin (1:100 enzyme/substrate) overnight at 37 °C. After digestion, the enzymatic reaction was terminated using 5% acetic acid.

The enrichment of phosphopeptides was performed using a gallium-based IMAC column (Pierce, Rockford, IL), according to manufacturer's protocols with minor modification. Briefly, about 100 µg of protein digest in 5% of acetic acid was loaded onto each IMAC column. After two washes with 0.1% acetic acid and two washes with 0.1% acetic acid plus 10% acetonitrile, the bound peptides were eluted four times with 20 µl of 100 mM ammonium bicarbonate, pH 9. The resulting eluate was acidified with 5% formic acid before mass spectrometry analysis.

Analysis of phosphopeptides by Multi-Dimensional Protein Identification Technology (MudPIT) and Linear Iontrap-Orbitrap

The eluted peptides from each IMAC column were analyzed by one MudPIT experiment, and each sample was analyzed in triplicate. As a quantitative control, digestions without phosphopeptide enrichment from either p1 or p45 $^{14}\text{N}/^{15}\text{N}$ mixture were also analyzed in triplicate. The MudPIT experiment was based on a previous method³⁸ with modifications tailored to phosphopeptide analysis. Peptides were pressure-loaded onto a 100-µm i.d. fused silica capillary column packed with a 5 cm long, 5 µm Partisphere strong cation exchanger (SCX, Whatman, Clifton, NJ) and a 5 cm, 5 µm Gemini C18 material (Phenomenex, Ventura, CA), with the SCX end fritted with immobilized Kasil 1624 (PQ Corporation, Valley forge, PA). After desalting, a 100-µm i.d. capillary with a 5-µm pulled tip packed with 15 cm 5-µm Jupiter C18 material was attached to the SCX end with a ZDV union, and the entire column was placed inline with an Eksigent pump (Eksigent Technologies, Dublin, CA) and analyzed using a 4-step separation. Three buffer solutions used were: 5% acetonitrile/0.1% formic acid (buffer A); 80% acetonitrile/0.1% formic acid (buffer B), and 500 mM ammonium acetate/5% acetonitrile/0.1% formic acid (buffer C). The first step consisted of a 100 min gradient from 0-100% buffer B. Steps 2-3 had the following profile: 3 min of 100% buffer A, 5 min of X% buffer C, a 10 min gradient from 0-15% buffer B, and a 130 min gradient from 15-45% buffer B, followed by a 20 min gradient increase to 100% buffer B, and a reverse of gradient to 100% buffer A. The 5 min buffer C percentages (X) were 30, 70% and 100% respectively. As peptides were eluted from the microcapillary column they were electrosprayed directly into a hybrid LTQ linear ion trap and Orbitrap (Thermo, San Jose, CA) with the application of a distal 2.4 kV spray voltage. A cycle of one full-scan with 60,000 resolution at 400 m/z by Orbitrap (400-1400 m/z) followed by five data-dependent MS^2 scan plus neutral loss-dependent MS^3 scan by LTQ was repeated continuously throughout each step of the multidimensional separation. Normalized collision energy of 35% was used while acquiring the MS^2 and MS^3 spectra.

Identification, quantification of phosphopeptides and phosphoproteins; bioinformatic analysis

MS^2 and MS^3 spectra were analyzed using the following software analysis protocol. Both spectra were searched with the ProLucid algorithm³⁹ against the EBI rat IPI database (<ftp://ftp.ebi.ac.uk/pub/databases/IPI/>, version 3.17, releasing date May 18, 2006), that was concatenated to a decoy database in which the sequence for each entry in the original database

was reversed. The search parameters include a static cysteine modification of 57.02146 amu and differential modification on serine, threonine and tyrosine residues of 79.9663 amu. Trypsin specificity was required for all peptides. The database search results were assembled and filtered using the DTASelect program with a spectra level false positive rate of less than 0.5%, mass accuracy of 5 ppm. Under such filtering conditions, the estimated false positive rate was below 1% at the peptide level and below 3% at the protein level.

The assembled search result file was used to obtain quantitative ratios between ^{14}N (sample) and ^{15}N (reference) using the software Censu⁴⁰. Censu allows users to filter peptide ratio measurements based on a correlation threshold because the correlation coefficient (values between zero and one) represents the quality of the correlation between the unlabeled and labeled chromatograms, and can be used to filter out poor quality measurements. In this study, only peptide ratios with correlation values greater than 0.5 were used for further analysis. Because both p1 and p45 cortices were mixed at 1:1 ratio with the common internal standard ^{15}N brain, the ratio difference between p1 and p45 ratios for the same phosphopeptide represents the difference in the phosphorylation level at that specific site in the protein. For singleton analysis, we required the $^{14}\text{N}/^{15}\text{N}$ ratio to be greater than 5.0 and the threshold score to be greater than 0.5. The threshold score ranges from zero to one and represents the quality of the singleton analysis with one being the most stringent. For any peptides that were assigned a $^{14}\text{N}/^{15}\text{N}$ ratio and determined to be singleton, we relied on the $^{14}\text{N}/^{15}\text{N}$ ratio and it was not considered a singleton. In addition, the resulting list of phosphopeptides was also validated using an automated phosphorylation validation program Debunker⁴¹. For Debunker filtering, we required a Debunker output score of at least 0.95 for a peptide for it to be considered as a validated phosphopeptide. In the end, the quantified and validated phosphopeptides and proteins were assembled as the final output.

For Gene Ontology (GO) analysis, annotations were obtained from www.geneontology.org. Almost all nuclear proteins were annotated with multiple molecular functions. For the construction of the pie graph (Figure 3C), the first molecular function was chosen.

The Pearson's correlation analysis between total and phosphorylated proteins was performed using Prism (San Diego, CA). The pathway analysis and protein-protein interaction network analysis was performed using Ingenuity Pathway Analysis software (Mountain View, CA).

Results

High confidence identification of phosphopeptides from brain nuclear extraction

The experimental procedure is outlined in Figure 1. To assess the general applicability of using ^{15}N enriched tissue as a common internal standard for quantitative phosphoproteomic measurements, as well as to survey the nuclear phosphorylation events associated with postnatal brain development, postnatal day one (p1) and postnatal day forty five (p45) rat brain cortical homogenates were mixed with a ^{15}N labeled p45 rat brain homogenate at a 1:1(wt/wt) ratio. Following a nuclear enrichment, the $^{14}\text{N}/^{15}\text{N}$ mixtures were digested with trypsin. The phosphopeptides were enriched using immobilized metal ion affinity chromatography (IMAC), and then analyzed by multi-dimensional protein identification technology (MudPIT) with neutral loss dependent MS³. The resulting spectra were searched with ProLucid³⁹, and filtered with DTASelect⁴² with a peptide false positive rate less than 1%. Finally, the peptides were quantified with Censu, which extracts the ^{14}N and ^{15}N chromatograms for each peptide and determines the $^{14}\text{N}/^{15}\text{N}$ ratio using linear regression analysis⁴³. Each preparation was analyzed in triplicate, and the results were pooled to generate the final output.

The dramatic enrichment of nuclear proteins was demonstrated by western blot analysis using an antibody against histone H3 comparing a total tissue homogenate and a nuclear enriched

preparation (Figure 2A). In contrast, the immunoreactivity of a synaptic marker (ionotropic glutamate receptor 2/3) showed a large decrease in the nuclear sample. From the MudPIT analysis, we identified 785 and 739 proteins resulting from 2614 and 2815 peptide identifications from the p1 and p45 samples, respectively. This represents identifications of both phospho- and non-phosphopeptides (Figure 2B). The p1 cortex yielded 551 proteins from 1523 unique phosphopeptides, and the p45 cortex yielded 560 proteins from 2121 unique phosphopeptides. Thus, phosphopeptides represented 58% and 75% of our identifications from p1 and p45 cortices, respectively. The relatively low percentage of phosphopeptides versus total (modified plus unmodified) peptides might be due to the highly complex nature of brain nuclear extract. Although this number of identified phosphorylated peptides is comparable with other reports in the brain^{32, 44}, each analysis of the same sample resulted in an increased number of phosphopeptides suggesting more analyses are required for a more complete phosphoproteome coverage of our complex sample (Figure S1). In addition, for more complete phosphoproteome coverage, it has been suggested that the combination of different phosphopeptide enrichment strategies is required⁴⁵. Several large-scale analyses of different phosphoproteomes have revealed a consistent trend of the relative abundance of Ser, Thr or Tyr phosphorylation sites^{15, 22, 31}. In our dataset, the distribution showed a similar relative abundance of 84.5%, 13.4%, and 2.1% for Ser, Thr and Tyr, respectively, in both p1 and p45 samples (Figure 2C). The relative ratios of singly, doubly and triply phosphorylated peptides is also in proximity with previous studies^{22, 31}, which were 74%, 23.6% and 2.4%, respectively (Figure 2D). Overall, our phosphopeptide identifications appear to be consistent with previous phosphoproteomic analyses.

During the collision activation dissociation (CID) process in an ion-trap mass spectrometer, phosphoserine and phosphothreonine frequently lose phosphoric acid while phosphotyrosine occasionally loses metaphosphoric acid. Thus, the appearance of fragment ions with an m/z 32, 49 or 98 less than the peptide precursor ions in MS² spectra strongly indicates that the peptide contains at least one phosphoserine, phosphothreonine residue. This neutral loss event is commonly used to trigger a data dependent MS³ in phosphopeptide analysis to increase both the number and confidence of identifications^{18, 46}. In our study, a prominent precursor ion neutral loss of 98, 49 or 32 in the MS² scan is selected for further fragmentation, and the fragmentation pattern appears in the MS³ spectra. As illustrated in Figure 2E, 71% and 80% of the phosphopeptides identified from MS³ spectra were also identified from MS² spectra, providing greater confidence for these phosphopeptides. To further increase our confidence in our phosphopeptide identifications, we applied a machine-learning program, Debunker⁴¹, to validate phosphopeptide identifications derived from the MS² spectra. Debunker uses a support vector machine binary classification to derive a probability score for phosphopeptide identifications from the CID spectra. Prominent spectral features such as neutral loss of precursor ions, neutral loss of fragment ions, and intensity of b or y ion series are incorporated to calculate a probability score as an assessment of the validity of the phosphopeptide identification. A predictive value from 0 to 1 is assigned to the possible phosphorylation event. A value less than 0.5 indicates that the phosphorylation prediction is negative, while a value greater than 0.5 indicates that the prediction is positive for a phosphorylation event. Values close to 1 are strongly predictive of a phosphorylation event. Requiring a predictive value greater than 0.95 in both samples, 86.5% of the phosphopeptides were determined as highly confident (Figure 2E).

The two sets of validated phosphopeptides were site-localized using a binomial probability approach similar to what has been described previously^{22, 47}. They were then separately analyzed by Motif-X algorithm⁴⁸ to extract phosphorylation sequence motifs. The default significance value was set to 10⁻⁶ for the sequence analysis and at least 20 occurrences were required to form the motif with the resulting motifs listed in Figure S2 and S3. Largely consistent with a previous analysis on HeLa cell nuclear proteome¹⁸, the major motifs

generated from either p1 or p45 cortices were proline directed and acidiphilic sites. Motifs between p1 and p45 cortices were similar except for a single motif, xPxxxKS(p)PxxxKx, which was found exclusively in p45 cortices. Among the proteins that contain this motif are neurofilament proteins (NFs). It has been shown that mitogen-activated protein kinases (Erk1, Erk2) preferentially recognize this motif⁴⁹. Phosphorylation of NFs by Erk1 and Erk2 at the KSP motif is thought to result in conformational change of sidearms of NF proteins and affect their association with each other and with microtubules. These phosphorylation events lead to the dynamic reorganization of neuronal cytoskeleton and thus the remodeling of synapse⁴⁹, a process that is predominantly seen in adult brain (p45).

Quantification of brain nuclear proteome in different developmental stages

Phosphopeptide ratios from either p1 or p45 cortices against the common ¹⁵N internal standard are plotted as histograms in Figure 3A. For p1 cortex, the range of magnitude in phosphopeptide ratios spans from 50 fold less to 10 fold more. In comparison, the range of magnitude for that of p45 cortices spans from 10 fold less to 10 fold more. Overall, we quantified 968 and 1674 phosphopeptides for the p1 and p45 cortices respectively, representing a quantification efficiency of 63.6% and 78.9% (Figure 3B and Table S1 for the complete list). The high confidence of our phosphopeptide identifications also extended to our quantified phosphopeptides. A high percentage (86.5%) of quantified MS² peptides was validated by Debunker with a probability score of 0.95 or greater. In addition, we quantified the peptides identified from MS³ spectra (Figure 3C and Table S2 for the complete list). Since Census uses peptide chromatograms generated from the MS spectra for quantification⁴³, the ratios of the quantified MS³ peptides were identical to the ratios of the MS² peptides from which they were derived. Furthermore, there were phosphopeptides quantified only with the MS³ spectra because the MS² fragmentation didn't generate enough sequence information to identify these peptides. Overall, 180 and 137 peptides (29% and 20% of all MS³ peptides) were quantified exclusively from MS³ identifications in p1 and p45 cortex, respectively.

Using Gene Ontology, we determined that 43% (p1 cortex) and 38% (p45 cortex) of the annotated quantified phosphoproteins had nuclear localization. In contrast, 17% of the proteins in the rat GO database are described as nuclear proteins indicating an enrichment of nuclear proteins supporting our western blot analysis. Corresponding to our western blot analysis, there were non-nuclear proteins in our sample. Also, it should be noted that Gene Ontology often annotates a protein with multiple localization, which reflects the literature. Thus, the percentage of nuclear proteins using the GO analysis may include proteins with that possess both cytoplasmic and nuclear localizations. The distribution of the molecular functions of these nuclear proteins was similar for the p1 and p45 samples and was combined in Figure 3C. Forty-eight percent of the quantified phosphoproteins were annotated to bind or regulate DNA. These were mostly proteins that regulated transcription, including beta-catenin, glucocorticoid receptor DNA-binding factor 1, and methyl-CpG-binding protein 2(MeCP2). Another large percentage of proteins were annotated to bind RNA or regulate RNA, including proteins that regulate RNA splicing. Numerous proteins involved in phosphorylation were quantified, and the majority of the proteins in this group were kinases, including protein kinase C (PKC), glycogen synthase kinase (GSK), 5'-AMP-activated protein kinase(AMPK), MAP/microtubule affinity-regulating kinase 2 (MARK2), and dual specificity tyrosine-phosphorylation-regulated kinase (Dyrk). In addition, phosphatases were also quantified within this functional protein group. Other functional categories quantified were structural proteins such as cytoskeletal proteins and nuclear pore proteins. Thus, we quantified many different functional classes of nuclear phosphoproteins.

It was impossible to derive a ¹⁴N/¹⁵N ratio for some phosphopeptides because a chromatographic peak for the corresponding ¹⁵N internal standard peptide was non-existent

or of extremely low signal. A large change in the abundance of phosphopeptide, however, has the greatest probability of indicating biological significance. This scenario has been designated as “singleton” indicating that only the ^{14}N peak was observed⁴³. Census can re-examine peptides that did not pass the original criteria for a high quality $^{14}\text{N}/^{15}\text{N}$ ratio for singleton peptides⁴³. We observed 191 ^{14}N singleton events in the p1 analysis and 102 ^{14}N singleton events in the p45 analysis. Since it is possible that the absence of a corresponding ^{15}N peak could indicate the ^{14}N peak is a misidentified peptide, we required singleton peptides to be annotated as nuclear proteins to be considered as true singleton peptides. With this additional requirement, we observed 56 ^{14}N singleton nuclear peptides in the p1 analysis and 8 ^{14}N singleton nuclear peptides from the p45 analysis (Table S3). We next examined the frequency of these singleton peptides within the three MudPIT analyses of each development time point. In the p1 cortex, one nuclear singleton peptide was observed in all three MudPIT analyses, six nuclear singleton peptides were observed in two of the MudPIT analyses, and the rest were only observed in one MudPIT analysis. In the p45 cortex, one of the nuclear singleton peptides was observed in two MudPIT analyses and the rest were observed in only one of the three MudPIT analyses. Since our ^{15}N internal standard was a p45 whole rat brain, the singleton peptides in the p1 analysis are potentially important phosphorylation events in neuronal development. Furthermore, it was expected that the p45 analysis would yield less singleton peptides since the sample and the internal standard were obtained from rats of the same age. The singleton peptides that were identified in the p45 analysis are most likely due to differences between whole brain and the cortical region, but we cannot rule out an isotopic effect of the ^{15}N labeling.

Clustering and functional classification of nuclear phosphoproteins

To further examine phosphoproteome changes during mouse brain development, we analyzed samples from p1 and p45 cortices using the identical protocol that has been described except we omitted the IMAC enrichment step. We will reference proteins from this analysis as “total” proteins (Table S5). These quantified proteins were employed to determine if the alterations observed with the phosphoproteins were due to changes in phosphorylation events or changes in protein expression. There were 194 proteins quantified in both p1 and p45 samples and both in total and phosphorylation enriched preparations (Table S4). A global comparison of the ratios (p1/p45) between phosphorylated and total proteins by Pearson’s correlation showed that there was statistically significant correlation between the two ratios (Figure 4A, $R=0.427$, $p<0.0001$).

These 194 proteins were then filtered based on the following criteria: a p1/p45 ratio of greater than 2 was considered as upregulated, while a ratio of less than 0.5 was considered as downregulated. The combinations of either up- or downregulation in either phosphoprotein or total protein categorize the proteins into 8 groups (Figure 4A and 4B). The majority of the phosphoprotein and total protein analyses were in agreement based on these criteria. In both phosphorylated and total protein analyses, 94 proteins showed no change, 35 proteins were observed to be downregulated in the p1 cortex, and 17 proteins were upregulated in the p1 cortex.

The remaining 48 proteins with differential changes between total and phosphoproteins suggest important protein phosphorylation regulation during cortical development. These proteins were further analyzed using the Ingenuity pathway analysis software (<http://www.ingenuity.com/>). Ingenuity uses Fisher’s exact test to identify significantly over represented functional categories in the input data against the Ingenuity Pathways Knowledge Base. With a significance cutoff value of 0.05 ($-\log(p)=1.301$), the analysis identified proteins in cell cycle, cellular assembly and organization, DNA replication, recombination and repair, as the top three significantly over represented categories (Figure 4C). Several other categories

including gene expression, cell morphology, developmental disorder, nervous system development and function were also significantly represented. Consistent with the Gene Ontology annotation results which showed an enrichment of nuclear proteins, all of these functional groups are intimately connected to the nucleus. We observed a high amount of overlap between these functional groups. For example, phosphoproteins involved in the regulation of the cell cycle were the largest common functional group with 14 proteins in this category, and all 10 proteins that were assigned to the category of DNA replication, recombination, and repair were also assigned to the cell cycle category. In addition, ten out of the 13 proteins assigned to the cell assembly and organization group were also assigned to the cell cycle category. This overlap likely stems from multiple biological functions assigned to these phosphoproteins.

Ingenuity also identified several protein-protein interaction modules, the top scored module was involved in cell cycle control, cell assembly and organization, as well as DNA replication, recombination and repair (Figure 4D). In this module, many proteins known to form the SWI/SNF chromatin remodeling complex, including SMARCA4 (Brg1), SMARCC2, polybromo 1 (PBRM1), chromodomain helicase DNA-binding protein 4 (CHD4), and remodeling and splicing factor (RSF1)^{50, 51}, were found to have upregulated phosphorylation events in p1 compared to p45 with Brg1 showing the greatest increase in phosphorylation at S1382. With the exception of transformer-2 alpha (TRA2A) and importin alpha 4 (KPNA3), the other 10 proteins identified in this network showed increase in phosphorylation in the p1 cortex. Therefore, it appears that many proteins controlling cell growth and chromatin remodeling are regulated by phosphorylation at specific sites in rat cortices during the early stage of brain development.

Regulated phosphorylation at different sites of the same protein

It is widely observed that different phosphorylation sites on the same protein can be differentially regulated. For example, it has been demonstrated that acute administration of the psychostimulant, amphetamine, causes an increase in the phosphorylation of the N-methyl-D-aspartate receptor NR1 subunit at serine 890 and 896, but not serine 897 in the brain⁵². As depicted in Figure 5A and 5B, two different proteins, heterogeneous nuclear ribonucleoprotein C (hnRNP C) and methyl-CpG-binding protein 2 (MeCP2), exhibited distinct abundance distribution patterns of phosphorylation at different residues. hnRNP C is a member of the heterogeneous nuclear ribonucleoproteins family of proteins that have been reported to be involved in DNA repair, telomere biogenesis, and gene expression⁵³. MeCP2 is a member of the family of methyl-CpG-binding domain proteins that function as long-range transcriptional repressors that mediate developmental silencing by binding to methylated DNA^{54, 55}. Phosphopeptides derived from hnRNP C showed both up- and downregulation in the p1 cortex, while phosphopeptides from MeCP2 showed either downregulation or no change in the p1 cortex. In contrast, peptides derived from total protein analysis of those two proteins showed much unanimous distribution pattern, either no change for hnRNP C, or downregulation of MeCP2 in the p1 cortex (Figure 5, C and D). We next plotted the distribution of standard deviation of the relative phosphoproteins ratios for all the proteins quantified in either the phosphorylated or total protein samples (Figure 5E). The distribution curves for standard deviation of quantified phosphoproteins appears to overlap with that of quantified total proteins with a few exceptions. This suggests that differentially regulated phosphorylation at different sites of the same protein is not a frequent occurrence in our quantitative analysis. However, it is possible that such differentially phosphorylated proteins may be below the limits of detection of the mass spectrometer or may be transient in nature preventing their detection.

Two specific examples of regulation of phosphorylation restricted to a discrete portion of a protein are shown in Figure 6. Figure 6A and 6B show a previously undocumented

phosphopeptide, MPFQAS(*p*)PGGK (S229), from the developmentally regulated transcription factor **MeCP2**. There was a slight increase of this peptide in p1 relative to p45 cortex. Figure 6C and 6D showed phosphorylation of two serine residues of MeCP2, AETS(*p*)ESSGSAPAVPEASAS(*p*)PK, with the second serine being designated as S80 by a previous study³¹. We observed dramatically less phosphorylation of this peptide in p1 cortex. Interestingly, there is almost no change between p1 and p45 cortex of another phosphopeptide, AGS(*p*)LES DGCPKEPAK (S421), a site previously reported to be phosphorylated in an neuronal activity dependent manner⁵⁶. As a reference, the peptide derived from total protein analysis revealed no changes between p1 and p45 cortex (Figure 6E and 6F). We also observed site-specific regulation of phosphorylation for the transcriptional regulator Brg1. Brg1 has been shown to play a critical role in mammalian neuronal differentiation⁵⁷. We found that for a documented phosphopeptide, EVDYSDS(*p*)LTEK (S1382)¹⁸, there was an over 15 fold increase in S1382 phosphorylation in p1 compared with p45 cortex (Figure 6G and 6H), but a much less (3 fold) increase in a peptide derived from total protein (Figure 6I and 6J), indicating a dramatic increase in phosphorylation at this site in early brain development, but a decrease when the brain approaches maturity. The MS² spectra of above mentioned phosphopeptides were shown in Figure S4. Thus, regulated phosphorylation at specific sites of these proteins may play important roles driving postnatal brain development.

Discussion

In this study, we used ¹⁵N labeled rat tissue as an internal standard combined with IMAC-based phosphopeptide enrichment to quantify protein phosphorylation in different stages of postnatal brain development. Our IMAC strategy consistently resulted in more than 50% of identifications being phosphopeptides facilitating the quantification of phosphopeptides. In total, we quantified over 1500 phosphopeptides in each sample giving rise to over 500 quantified phosphoproteins. Additionally, by clustering the quantified nuclear phosphoproteins based on their abundance of phosphorylation, we found that there was a significant correlation between the phosphorylation level changes and the total protein expression changes from different developmental stages of rat cortex. Finally, we discovered regulated, site-specific phosphorylation changes in two important transcriptional regulators, MeCP2 and Brg1.

Even with phosphopeptide enrichment strategies, the low stoichiometry of phosphorylation events often limits the identification of a phosphoprotein to one peptide. We have applied several strategies, however, to achieve high confidence in our identification and quantification of phosphopeptides. First, a decoy database search strategy⁵⁸ was used to control the false positive rate at peptide level to be below 1%. Second, after the identification of peptides, a support vector machine algorithm, Debunker, was applied to derive a binary classifier for each peptide filtering out peptides from low-quality spectra and the spectra lacking phosphopeptide fragmentation patterns commonly observed in CID⁴¹. In this study, only a small fraction of identified phosphopeptides (<15%) were filtered out based on stringent criteria. Third, using an Orbitrap mass analyzer to acquire MS spectra enabled us to distinguish phosphorylation from other modifications of similar mass. For example, protein sulfonation at each serine or threonine residue results in a molecular mass increment of 79.9568⁵⁹, a mass shift that is 0.0095 amu different from that of phosphorylation which can be distinguished for peptides that are under 1900 Da by Orbitrap with a 5 ppm accuracy. In addition, the increased accuracy of peptide precursor ion measurement greatly improves the quantification using extracted chromatograms⁶⁰. Finally, neutral loss dependent acquisition of MS³ spectra was applied to further increase the confidence of the identifications. A low percentage (~20%) of MS² identifications were confirmed by MS³, which is consistent with previous observations^{18, 61}. There are several potential reasons as to why a phosphopeptide may be identified only from the MS² scan. It is most likely that the number of fragment ions in the MS³ scan is not large enough to identify a phosphopeptide due to the insufficient trapping of the neutral loss peptide

ions. Alternatively, an MS³ scan event may not be triggered when a phosphopeptide analyzed by an MS² scan does not undergo complete neutral loss of phosphate under CID³², or proline-directed fragmentation in MS² generates ions that are more abundant than the neutral loss peptide ions. However, over 70% of the identifications generated from MS³ spectra matched that of MS² spectra. The remaining, high-quality identifications from MS³ spectra thus provided additional identifications, and these can increase the number of quantified phosphopeptides as a whole. The mechanism underlying phosphopeptides identified solely from MS³ scans is that CID in MS² may generate a dominant neutral loss peptide ion and less or undetectable fragment ions, which leads to a complete lack of peptide identifications or the identification of an incorrect phosphopeptide. Thus, employing MS³ analysis, Debunker, decoy database searching, and a high resolution and high mass accuracy mass spectrometer generate a particularly confident quantified phosphoprotein analysis.

A previous report performed global mass spectrometry analysis of nuclear phosphoproteins from a HeLa cell line¹⁸. Although different nuclear extract methods were performed, this report also identified greater than 50% contaminant or unknown proteins. A direct comparison of individual phosphoproteins between these studies is difficult, because our starting material consisted of multiple rat cell types embedded within extracellular matrices, while the sample of this previous report consisted of a single human cell type cultured in vitro. In addition, different phosphopeptide enrichment strategies were employed between the two studies. However, both reports demonstrated the identification of a large percentage of DNA binding proteins, kinases, and proteins involved in protein splicing. Furthermore, both reports identified proline-directed and acidophilic kinase motifs as the major motif identified suggesting this dominant nuclear phosphorylation. In addition to phosphopeptide identification, our study focused on quantification of phosphopeptides. We have previously employed SILAM analysis to quantify proteins from both liver and brain tissues^{33, 35}. In the brain analysis, we identified changes in protein expression during postnatal development, and over 50% of the annotated proteins quantified have been previously described in the literature using other quantification methods³⁵. Nevertheless, quantification of phosphoproteins provides unique challenges to mass spectrometry. The largest challenge is that phosphorylation sites can be differentially regulated on the same protein, as demonstrated by our analysis of MeCP2 and Brg1. Although many laboratories have successfully performed large-scale phosphoprotein quantification, there are very few laboratories that have combined the complexity of mammalian tissue with quantitative phosphorylation analysis. One report quantified a small number of phosphoproteins from brain³¹, while more recently in another report, the iTRAQ strategy was employed to quantify 1339 phosphorylation sites from the post-synaptic density (PSD) from brain³². iTRAQ labels and mixes peptides after the isolation of the PSD and trypsin digestion, while using metabolically labeling, the ¹⁴N and ¹⁵N samples are mixed before fractionation, which reduces the accumulation of the systematic errors during sample preparation. Because of differences in phosphopeptide enrichment strategies and mass spectrometers employed, it is difficult to compare quantitative phosphoproteome analysis using SILAM to the analysis used by Trinidad et al., but future studies directly comparing these two strategies would be valuable.

Our results also highlight the importance of using an unmodified protein ratio as a control to interpret the phosphoprotein ratio changes. We observed the majority of the phosphopeptides have a similar expression pattern as the unmodified peptides from the same protein. Among the proteins quantified with both phosphorylation ratios and total ratios, over 70% of the proteins were either unchanged or the change in expression occurred concurrently. This is in contrast with EGF signaling, where site-specific phosphorylation levels frequently changed without total protein changes²². This might be the result of the different systems analyzed in these two studies. First, the study of EGF signaling was performed on a less complex system, mammalian cell culture, and under EGF stimulation to achieve signal transduction, while we

were comparing the differences in protein phosphorylation in brain tissues between two developmental stages. Furthermore, in the EGF signaling cascade, the entire signal transduction occurs rapidly in the time scale of minutes. While in our study of postnatal brain development, the signaling event happens in the time scale of weeks⁶² allowing the steady growth of neurons and glia to increase the brain mass. It is therefore conceivable that many important signaling proteins are up- or down regulated as a whole together with the phosphorylation of specific sites. In addition, Trinidad et al³² observed similar trends between protein expression and phosphorylation in their analysis of brain tissue. Thus, this indicates that the biological significance of many of the phosphorylation events we quantified is equally crucial to the p1 cortex as to the p45 cortex.

Alterations in the expression of phosphopeptides between p1 and p45 samples, however, did not always correspond to the alterations in the expression of the unmodified peptides. For example, the average p1/p45 ratio of the phosphopeptides from the Brg1 protein was 7.5 while the average p1/p45 ratio of the unmodified peptides was 1.7 suggesting a large increase in phosphorylation of Brg1 at p1 compared to p45. Alternatively, the unmodified Rap1-interacting factor 1 (RIF1) expression was low (-5.9) at p1 compared to p45 while the phosphopeptides of RIF1 were relatively unchanged (1.85) between the p1 and p45 samples indicating more phosphorylation at p1 compared to p45. These observations suggest that these phosphorylation events are important for cellular processes early in postnatal brain development. Previous reports examining individual phosphorylation events have also demonstrated changes in protein phosphorylation during brain development⁶³⁻⁶⁵. For example, it has been reported that in juvenile mouse cortices, visual stimulation induces the phosphorylation of mitogen- and stress-activated kinase (MSK), cAMP responsive element-binding protein (CREB), and histone H3, while this induced phosphorylation of CREB and H3, but not MSK, was greatly reduced in adult mouse cortices⁶⁵. Therefore, we have identified novel regulated phosphorylation on known sites that could provide further understanding into the signaling of the developing cortex.

Cerebral cortical development is a dynamic process that requires complex but precise coordination of cell proliferation, death, migration, differentiation, dendritic arborization, and synaptogenesis. Many of the phosphorylation signaling pathways are involved in this process, including multiple mitogen-activated protein kinase pathways and the Wnt pathway^{66, 67}. These pathways are activated by a variety of extracellular growth factors and result in the phosphorylation of nuclear proteins to alter transcription. It is still unknown, however, how these multiple phosphorylation signaling pathways work together to promote the precise coordination of the numerous biological processes during development. Adding to the complexity is that the cortex consists of multiple different types of neurons in addition to different types of glial cells, and the localization of specific growth factor receptors can vary dramatically between different cortical regions⁶⁸. Thus, our analysis most likely represents global or the most abundant nuclear changes in phosphorylation found throughout the cortex. By using Ingenuity pathway analysis and the literature, we observed that many of the proteins forming the chromatin remodeling complex, and proteins that interact with the complex had an increase in phosphorylation in the p1 cortex. Neurogenesis peaks at birth for most neurons in rat brain, and synaptogenesis peaks between p14 and p21⁶². The phosphorylation of Brg1 and other related proteins might play an important role in these processes. Brg1 is a component of the SWI/SNF chromatin remodeling complex with an evolutionarily conserved DNA-dependent ATPase domain⁶⁹. Consistent with our findings, previous studies have shown the requirement of Brg1 phosphorylation in mitotic cells for the SWI/SNF complex to be excluded from condensed chromatin, and this might be part of the mechanism leading to transcriptional arrest during mitosis⁷⁰. It was later shown that Brg1 is required for neural differentiation in mammals through interaction with Ngnr1 and NeuroD transcription factors⁵⁷. Chromatin remodeling, DNA modification and histone modification is known to play a crucial role in

activity-dependent dendritic development and neuronal plasticity^{71, 72}. A recent study demonstrated that the neuronal chromatin-remodeling Brg/Brm-associated factor (nBAF) complex promotes dendritic morphogenesis, and that the process involves a combinatorial selection of an actin-interacting protein BAF53b and requires Brg/Brm ATPase activation⁷³. Therefore, our finding of increased phosphorylation at specific sites of Brg1 and related proteins will likely provide further insights for the understanding of how neuronal activity lead to the control of dendritic growth, neurogenesis and therefore brain development.

Neurons and glia are two main classes of cells making up the nervous system, and both encompass many specialized sub-types. As these cells exit the cell cycle, they migrate and differentiate establishing the form of the nervous system. In humans, these processes occur between the third trimester and the first years of life. In the rat, these same processes are condensed into the first month of postnatal life, and thus, generate an experimental model for human brain development. The molecular mechanisms underlying human brain development are not fully elucidated, but further research can provide potential therapies for poorly understood neurodevelopmental disorders, such as Rett syndrome, autism, and schizophrenia⁷⁴⁻⁷⁶. In Rett syndrome for example, mutations in MeCP2 have been demonstrated to cause the majority of the cases, but how these mutations cause the deleterious phenotype is unknown⁷⁵. One report suggests that the phosphorylation of MeCP2 plays a role in progression of the disease⁵⁶. Since phosphorylation plays an essential role in neural activity, global quantitative phosphorylation analysis becomes indispensable for neuroscience research. For the first time, we demonstrate the value of employing a metabolically labeled rat tissue for global quantitative phosphorylation analysis of brain tissue and highlight the importance of phosphorylation events in neuronal development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

An outline of the experiment. Postnatal day one (p1) and postnatal day forty five (p45) rat cortical homogenates were mixed with a ^{15}N p45 whole rat brain homogenate at a 1:1 ratio. Following a nuclear enriched preparation, the $^{14}\text{N}/^{15}\text{N}$ mixtures were digested with trypsin. Half of the digests was subject to phosphopeptide enrichment using IMAC, and then analyzed by MudPIT with neutral loss dependent MS^3 . The other half of the digests was directly analyzed by MudPIT. The resulting spectra were searched with ProLucid and filtered with DTASelect, and then, the identified peptides were quantified with Census. Each preparation was analyzed in triplicate and the results were pooled to generate the final output.

Figure 2.

Phosphopeptide identification. (A) Western blot analysis using antibodies against a nuclear marker (histone H3) and a synaptic marker (GluR2/3). Twenty-five micrograms of total cortical lysate and the nuclear enriched preparation from a p45 rat were compared. (B) A comparison of total protein/peptide identifications and phosphoprotein and phosphopeptide identifications from the p1 and p45 cortex. (C) Relative abundance of serine (pS), threonine (pT) and tyrosine (pY) phosphorylated peptides in p1 and p45 cortex. (D) Distribution of singly, doubly and triply phosphorylated peptides in the p1 and p45 cortex. (E) table shows respectively the number of phosphopeptides identified from MS², MS³ spectra, the number of overlapped phosphopeptides identified from both MS² and MS³ spectra, percent of phosphopeptides identified solely from MS³ spectra, and the number and the percent of identified phosphopeptides that were validated using Debunker.

Figure 3.

Quantification phosphopeptides. (A) Histograms of log transformed phosphopeptide ratios between p1 or p45 cortices and a common internal standard (^{15}N labeled rat whole brain). (B) Number of peptides quantified and Debunker validated phosphopeptides identified in MS^2 . (C) Number of peptides quantified and Debunker validated phosphopeptides identified in MS^3 . (D) GO annotation of quantified phosphoproteins in the p1 and p45 cortex using the category of molecular function.

Figure 4. Clustering of phosphoproteins. (A) Correlation of p1/p45 ratios between total protein and phosphorylated protein. Proteins are clustered based on eight categories depending on the changes of either total or phosphorylated protein ratios, with different colors indicating different clusters (See Table S4 for color code). R-value was calculated using Pearson's correlation ($p < 0.0001$). (B) Number of proteins in each of the eight categories. With respect to p1/p45 ratio, 1 indicates a ratio change of more than or equal to two fold, -1 indicates a ratio change of less than or equal to 0.5, while 0 indicates the ratio was between 0.5 and 2. (C) Ingenuity analysis of significantly changed functional categories using Fisher's exact test. (D) Protein-protein interaction network that represent the function of cell cycle and DNA replication, recombination and repair. Red indicates an increased level of protein phosphorylation and green indicates a decreased level of phosphorylation.

Figure 5.

Phosphorylation levels are regulated at different sites for a subset of proteins. Shown are distribution of quantification ratios of multiple phosphopeptides with sites indicated (A) and multiple unmodified peptides (B), for a protein hnRNP C. (C and D) Similar to (A and B) except that the ratio distributions shown are for MeCP2. (E) Distribution of standard deviation of protein ratios calculated by Census. Here the standard deviation is the precision of peptide ratio measurements that are used to calculate protein ratio. The curve shows the percentage of proteins from either total proteins or phosphoproteins in each interval of standard deviation values.

Figure 6.

Reconstructed chromatograms for peptides from two selected proteins. (A through D) shows peptide precursor ion chromatograms (blue) for two different phosphopeptides of MeCP2 from either p1 or p45 cortex, against that of the common internal standard (red). The calculated ratio (sample/reference) by Census is shown in each chromatogram. The chromatograms for an unmodified peptide from either p1 or p45 are shown in E and F. (G and H) Chromatograms of a phosphopeptide from Brg1 shows ratios of either p1 or p45 against the ¹⁵N peptide, while the p1 or p45 ratios of an unmodified peptide from Brg1 of either against the ¹⁵N peptide are shown in I and J. The phosphorylation site was indicated as (p).