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¹⁵N labeled brain enables quantification of proteome and phosphoproteome in cultured primary neurons

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Abstract

Terminally differentiated primary cells represent a valuable *in vitro* model to study signaling events associated within a specific tissue. Quantitative proteomic methods using metabolic labeling in primary cells encounter labeling efficiency issues hindering the use of these cells. Here we developed a method to quantify the proteome and phosphoproteome of cultured neurons using ¹⁵N labeled brain tissue as an internal standard, and applied this method to determine how an inhibitor of an excitatory neural transmitter receptor, phencyclidine (PCP), affects the global phosphoproteome of cortical neurons. We identified over 10,000 phosphopeptides and made accurate quantitative measurements of the neuronal phosphoproteome after neuronal inhibition. We show that short PCP treatments lead to changes in phosphorylation for 7% of neuronal phosphopeptides and that prolonged PCP treatment alters the total levels of several proteins essential for synaptic transmission and plasticity and leads to a massive reduction in the synaptic strength of inhibitory synapses. The results provide valuable insights into the dynamics of molecular networks implicated in PCP-mediated NMDA receptor inhibition and sensorimotor deficits.

Keywords

mass spectrometry; phencyclidine; phosphorylation; quantification; stable isotope labeling

Introduction

Quantitative proteomics and phosphoproteomics using stable-isotope labeling (SIL) in cells have been widely used in cell-based studies^{1, 2}. The use of SIL in postmitotic, terminally differentiated cells including primary neurons, cardiac myocytes, osteoclasts, has serious limitations³. In postmitotic cells, incorporation of heavy isotopes into proteins is restricted to actively synthesized proteins and thus heterogeneously labeled proteins create a poor reference standard and inaccurate quantitation. To address this issue, primary cells can be labeled starting from progenitor cells and maintained in heavy media until differentiation⁴. An alternative approach pioneered by Ishihama et al is to use a cultured cell line grown with

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Data Availability

The raw mass spectrometry data is available at: <http://fields.scripps.edu/published/lliaopcp2011/pcp-n15/>

heavy isotopes for use as a spike-in internal standard to quantify tissue proteome^{5, 6}. A potential limitation to this approach occurs when the cell type used as an internal standard does not express proteins present in the tissue. We previously developed a method for stable-isotope labeling in mammals (SILAM) to quantitatively measure the proteome and phosphoproteome changes in rat brain during development⁷⁻⁹. SILAM labels all amino acids with ¹⁵N in proteins of the organism providing an internal standard for all proteins in the tissue under study. Here, we tested the idea of using ¹⁵N enriched whole brain tissue as a common internal standard using the ratiometric (ratio of ratios) approach developed by MacCoss et al¹⁰ to quantify the proteome and phosphoproteome in primary cultured cortical neurons after perturbation with the psychotomimetic drug phencyclidine (PCP).

In humans, PCP induces both positive symptoms of schizophrenia, such as delusion, hallucination and disorganized thoughts; and negative symptoms such as loss of motivation and depression¹¹. PCP mediated disruption of pre-pulse inhibition at the prefrontal cortex in rodents is a common model for a sensorimotor gating deficit frequently found in schizophrenia patients¹². The primary pharmacological effect of PCP is to block the activation of NMDA receptors¹³ and thus reducing Ca²⁺ entry. Among the plethora of signaling cascades activated by elevated levels of postsynaptic Ca²⁺, Calcium-Calmodulin dependent kinase II (CaMK2) dependent phosphorylation of multiple targets lead to changes in gene expression and induces multiple forms of synaptic plasticity essential for learning and memory. Many of the signaling events intimately involve the binary switch between phosphorylation and dephosphorylation of a large number of effector molecules^{14, 15}. Global quantitative analysis of protein phosphorylation would likely provide new insights on the molecular details of the signaling events. However, due to the low stoichiometry and dynamic nature of protein phosphorylation, it is necessary to enrich phosphorylated species for mass spectrometry identification and quantification. Well established enrichment strategies include strong-cation exchange (SCX) followed by immobilized metal ion chromatography (IMAC)¹⁶, SCX followed by TiO₂ affinity capture^{17,18}, hydrophilic interaction chromatography (HILIC) followed by IMAC¹⁹, as well as enrichment through chemical derivatization²⁰. These methods differ in their selectivity for phosphopeptides and the requirement for the starting sample amount. We used a combination of HILIC-IMAC for tandem enrichment because it offers an optimal balance of ease of use, reproducibility, and compatibility with small sample sizes (~1 mg level).

In this study, we show that although many proteins in SILAM brain express with different levels as those in primary neurons, we were able to accurately quantify over two thirds of the proteins identified in neurons. Moreover, quantification of the phosphoproteome using SILAM brain is as accurate as quantification of the neuronal proteome. By using the SILAM brain in conjunction with the HILIC-IMAC approach to phosphoprotein enrichment, we were able to quantify phosphoproteome changes in primary cortical neurons after treatment with PCP. Changes in phosphorylation of membrane receptors may lead to the changes in their functional properties²¹, consequently we used electrophysiology to test one of the proteins with observed phosphorylation changes and identified changes in GABAergic inhibitory neural transmission after PCP mediated neuronal perturbation.

Experimental Section

Culture of primary cortical neurons and processing of samples

Neocortices from embryonic day 18 rat were dissected, dissociated, and neurons cultured in Neurobasal media (Invitrogen, Calsbad, CA) based on a previously described protocol with minor modifications²². Briefly, the neurons were plated at a density of 60,000 cells/cm² and maintained in Neurobasal media supplemented with B27, penicillin (50 µg/ml), streptomycin (50 U/ml), and glutamine (2 mM). After 14 days in culture, neurons were

collected with HEPES-buffered sucrose (10 mM HEPES, pH 7.4, 0.32 M sucrose, protease inhibitor cocktails (Roche, Mannheim, Germany), 2 mM NaF, 1 mM Na₃VO₄), then homogenized and centrifuged at 700 × g for 10 minutes to remove the nuclei and cell debris. The resulting supernatant fraction (S1) was used for further analysis.

For SILAC experiments, cortices from embryonic day rats were dissected, dissociated, and neurons were plated at a density of 3,000,000 cells/10 cm² dish in Neurobasal media (Invitrogen, Calsbad, CA). The media were supplied with either heavy isotope (¹³C¹⁵N)-enriched arginine and lysine (Spectra Isotopes, Cambridge, MA), or light isotope (¹²C¹⁴N)-enriched arginine and lysine (Sigma, St. Louis, MO). After the 14 days in culture, the light neurons were untreated while the heavy neurons were treated with PCP. In another experiment, the treatment strategy was inverted. After treatment, neurons were immediately collected with the same HEPES-buffered sucrose as used for SILAM experiments, and then used for further analysis.

For crude synaptosome preparation, cultured cortical neurons were collected with the same HEPES-buffered sucrose solution, and centrifuge at 700 × g for 15 minutes. The supernatant were centrifuged at 10,000 × g for 15 minutes, and the resulting pellet (P2) was used as the synaptosome enriched membrane fraction.

Phosphopeptide enrichment combining Hydrophilic Interaction Chromatography (HILIC) and Immobilized Metal Affinity Chromatography (IMAC)

One milligram of soluble cytosolic fraction (S1) were mixed with 1 mg of S1 from ¹⁵N labeled rat whole brain homogenate, and were precipitated with cold acetone at −20°C overnight. The precipitates were centrifuged at 14,000 × g for 15 minutes at 4°C. The pellets were solubilized and reduced with 100 mM Tris-HCl/8 M urea/5 mM DTT 100 µl, cysteine alkylated with 10 mM iodoacetamide, and digested with 20 µg of trypsin at 37°C overnight. The digestion was terminated by adding TFA to 0.4%. The resulting peptide solution was desalted with SepPak cartridge (Waters, #WAT054955, 100 mg of tC18 beads for 2.5 mg digest) according to the manufacturer's instructions, and lyophilized for HILIC separation. The HILIC separation was on a 13071TSKgel Amide-80 column (5 µm, 4.6×250 mm) from TOSOH Biosciences. HILIC separation was based on a previously described method²³. The gradient was started with 80% of HILIC buffer B (98% acetonitrile with 0.1% TFA, buffer A composed of 98% water and 0.1% TFA) running at 0.5 ml/min. The separation gradient is as follows: the constant flow is set at 0.5 ml/min, and followed by 5 minutes 80% B, 40 minutes 80–60% B, 5 minutes 60–10% B, 5 minutes 10% B, and 5 minutes 80% B. Fractions were collected every 5 minutes from the start of the gradient until 55 minutes and a total of 11 fractions were collected. All fractions were snap-frozen in liquid nitrogen and lyophilized. Each fraction was dissolved in 400 µl of IMAC sample buffer (250 mM acetic acid/40% acetonitrile, pH 2.5–3.0), and phosphopeptides were enriched with PHOS-Select Iron Affinity Gel slurry (Sigma, St. Louis) based on a published method¹⁶ with minor modifications, and eluted with 400 mM ammonium hydroxide. The 11 phosphopeptide enriched fractions were combined and ready for mass spectrometry analysis.

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

For each combined phosphopeptide sample, two 6-step MudPIT²⁴ experiments for each combined sample were performed to maximize the coverage. Peptides were pressure-loaded onto a 100 µm i.d. fused silica capillary column packed with strong cation exchanger (SCX, Whatman, Clifton, NJ) and a C18 material (Phenomenex, Ventura, CA), with the SCX end fritted with immobilized Kasil 1624 (PQ Corporation, Valley forge, PA). After desalting, an analytical column of 100-µm i.d. capillary packed with another C18 material can be attached

to the SCX end with a ZDV union, and the column will be placed in line with a HPLC pump as a nanospray ionization source, and interfaced with an LTQ-Orbitrap mass analyzer. Three buffer solutions are generally used: 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. Each of the remaining steps are composed of a 5 min of step-increased salt buffer (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. As peptides were eluted from the microcapillary column they were electrosprayed directly into the LTQ-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/MS scan plus neutral loss-dependent MS/MS/MS scan by LTQ were repeated continuously throughout each step of the multidimensional separation.

Identification, quantification of phosphopeptides and phosphoproteins

The MS/MS spectra were searched with the ProLucid algorithm developed in Yates lab²⁵ against an IPI rat database (Version 3.30, released at June 28, 2007: <ftp://ftp.ebi.ac.uk/pub/databases/IPI/old/RAT/>) that was concatenated to a decoy database in which the sequence for each entry in the original database was reversed. The introduction of decoy database was used to assess the false discovery rate of spectra-database matching algorithm²⁶. To identify phosphopeptides, the search parameters included a differential modification on serine, threonine and tyrosine residues of 79.9663 amu, indicating the addition of phosphorous group(s) on those residues. The database search results were assembled and filtered using the DTASelect program²⁷.

The assembled database matching result file was used to obtain quantitative ratios between the ¹⁴N and ¹⁵N version of each peptide using the software Census²⁸. Because the control and PCP-treated neurons were individually mixed with ¹⁵N brain homogenate, the ratio difference between control and PCP-treated sample for the same phosphopeptide represented the difference in the phosphorylation level at the identified site. For SILAC experiments, the peptide ratios between the light and heavy isotopes were directly used as surrogates for protein expression ratios between the control and PCP-treated neurons.

MRM validation of phosphopeptide ratios

Ten heavy (¹³C¹⁵N) arginine or lysine-containing phosphopeptides were synthesized (JPT peptide technologies, Berlin, Germany) and mixed with equal molar ratios, the mixture was used as spike-in internal standards. Three batches of neurons were either untreated or treated with PCP, 100 ug of cells lysate were spiked with 50 fmol of the ten heavy standards. Five transition ions for each peptide were selected for SRM, and the transition tables are listed in Table S5. A standard LC-SRM experiment were performed with a 90 minute gradient delivered by a Dionex nano-HPLC (Thermo) pump with a flow rate of 300 nl/min. SRM were performed on a Thermo TSQ-Vantage Triple Quadrupole mass spectrometer (Thermo, San Jose, CA). The nano-electrospray ionization conditions were similar to MudPIT experiments, while the TSQ conditions were set as: Q1 peak width was 0.7(FWHM), Q3 scan width was 0.002 mass unit, and the collision gass pressure was at 1.2 mTor.

Statistical and bioinformatics analysis

Two batches of the experiment were performed to generate biological replicates. To assess the reproducibility of mass spectrometry measurement, two MudPIT runs were performed in the first batch of samples. To test the significance of changed phosphorylation events, ANOVA was used when the phosphopeptides were quantified in both biological replicates.

Otherwise, Grubbs outlier test was applied to find significant outliers. In both cases the significance value used was $P < 0.05$.

To confidently assign the phosphorylation sites, a binomial probability based algorithm (A-score) was used²⁹. Only phosphorylation sites with $P < 0.05$ were further considered for motif analysis. For these phosphorylation sites, Motif-X algorithm³⁰ was applied to extract amino acid sequence patterns flanking the phosphorylation sites. We allow the occurrences of the same pattern to be at least 20 times, and a significance value to be 0.000001, to be considered as motifs. The IPI rat protein database was used as the background reference database.

Hierarchical clustering was applied to group phosphorylation sites with spectral counts of each site as the intensity matrix, using the freely available program Cluster 3.0³¹. The heatmap representation of the cluster was displayed using Java Tree View (<http://jtreeview.sourceforge.net/>).

Pathway analysis was performed using the commercially available software Ingenuity. Phosphoproteins with changed phosphorylation sites were mapped into the signal pathways in Ingenuity knowledgebase. As a control, we randomly generated the same number of phosphoproteins from the list of proteins with no change in phosphorylation, and mapped into the signal pathways in Ingenuity with identical parameters.

Immunoblotting

For immunoblot analysis, 30 μ g of protein from either total neuronal lysate or synaptosomal membrane preparation from primary cortical neurons was used. Western blot analyses were performed on samples from at three separate experiments. All antibodies were obtained from commercial sources as listed: The blots were scanned and band intensities were analyzed using AlphaEaseFC (Alpha Innotech, San Leandro, CA), followed by Student-t test to assess the statistical significance.

Electrophysiology

Isolation of primary neurons, neuron culture, and electrophysiological recordings of synaptic transmission were performed essentially as described (Maximov et al, Science 2009). Briefly, the cortexes were dissected from the brains of P1 mouse pups and neurons were dissociated by trypsin digestion and plated onto Matrigel-coated circle glass coverslips. Neurons were maintained in MEM medium supplemented with B-27, Glucose, Transferrin and AraC and analyzed at 14–16 days in vitro. Evoked synaptic responses were triggered by 1 ms, 0.9 μ A current injection through a local extracellular electrode (FHC Concentric Bipolar electrode) and recorded in whole cell mode using a Multiclamp 700B amplifier (Molecular Devices). In some cultures, 50 μ M PCP was applied to the neurons for either 1 hour or 3 hours before recording, and the same concentration of PCP was maintained in the recording buffer. For the washout experiments, the perfusion buffer was replaced with recording buffer without PCP for 15 minutes before the recording. The whole cell pipette solution contained (in mM): CsCl 135, HEPES 10, EGTA 1, Mg-ATP 4, Na4GTP 0.4, and QX-314 10, pH 7.4. The bath solution contained (in mM): NaCl 140, KCl 5, CaCl₂ 2, MgCl₂ 2, HEPES 10, and glucose 10, pH 7.4. Inhibitory transmission was isolated by addition of 50 μ M AP5 and 20 μ M CNQX to the bath solution. Data were recorded and analyzed with Clampfit 10.2 software (Molecular Devices). In total, three batches of cultures and between 20 and 30 neurons were recorded for statistical analysis.

Results

SILAM-based method to quantify proteome in primary neurons

We explored a strategy using SILAM rat brain to quantify proteome and phosphoproteome changes in cultured primary neurons after perturbation with PCP (Figure 1A). Because most symptoms of schizophrenia are ascribed to changes in the frontal cortex, we cultured cortical neurons from E18 rat embryos. Primary neurons maintained for 14 days *in vitro* (DIV) were treated with 50 μ M of PCP for 15 minutes in order to capture changes in protein phosphorylation. Homogenates from each population of neurons were mixed with ^{15}N -enriched rat brain (postnatal day 45) homogenate at a 1:1(w/w) protein ratio, and the 700 $\times$ g supernatant that was largely devoid of nuclei and cell debris was used for the entire analysis (designated as cytosolic fraction). A part of the cytosolic fraction of the mixture was analyzed by MudPIT²⁴ to identify and quantify the neuronal proteome. The remaining sample was fractionated by hydrophilic interaction chromatography (HILIC)¹⁹. Phosphopeptides were further enriched from each fraction using immobilized metal ion affinity chromatography (IMAC)¹⁶, and the phosphopeptide-enriched fractions were pooled and analyzed by MudPIT to identify and quantify the phosphoproteome. The ratio between ^{14}N and ^{15}N version of each phosphopeptide is calculated, and the ratio differences between phosphopeptides from either the control neurons or the PCP treated neurons is the measurement for phosphorylation changes¹⁰.

Overall, we expect neuronal signaling downstream of NMDA receptors to be inhibited by PCP. Fifteen minutes PCP treatments of neurons did not lead to any change in either the total protein levels or the phosphorylation state of S896 of the NR1 subunit of NMDA receptor. Several other proteins known to mediate activity-dependent remodeling of the excitatory synapse, including glycogen synthase kinase 3 β and β -catenin, also showed no change in protein as well as phosphoprotein levels (Figure S1A). In contrast, c-Fos, an immediate early gene that is transcriptionally upregulated upon neuronal activation³² - and that is also transcriptionally upregulated in prefrontal cortex of rats after localized PCP injection¹³ - showed upregulation in a dose-dependent manner (Figure S1B). Consistent with previous reports, we also observed a dramatic reduction of T202/Y204 phosphorylation of the extracellular-signal-regulated kinase (ERK1/2) without reduction of total ERK1/2 levels (Figure 1B)³³. ERK1/2 is the signaling node that is downstream of NMDA receptor activation and calcium entry³⁴, and phosphorylation of ERK1/2 at T202/Y204 increases ERK kinase activity. Our results indicate that short-term treatment of cortical neurons with PCP perturbed the neurons by inhibiting NMDA receptor mediated excitatory neural transmission.

The reproducible change in phosphorylation of the ERK1/2 kinase mediated by PCP on neurons allows us to explore the global effect on protein phosphorylation. As a reference point, we first examined global protein levels, with the expectation that most of the proteins will not change after a short treatment. Two replicate MudPIT analyses of either the control or PCP-treated neurons mixed with ^{15}N enriched brain lead to the identification of 6,306 ^{14}N proteins representing primary cortical neuronal proteins from an average of 14,000 peptides, and 3,944 ^{15}N proteins representing brain proteins from an average of 6,000 peptides. The dramatically lower number of identifications from the ^{15}N tissue is reproducible, and it is the combined effect of the complexity of brain tissue and a wider distribution of ^{15}N isotope envelopes in the mass spectra. There were 3514 proteins overlapping between primary neuronal and brain proteins (Figure 1C). We classified proteins identified exclusively from ^{14}N search (neuron only), those exclusively from ^{15}N search (brain only) and those with overlapped identifications (both) and performed Gene Ontology analysis (Figure 1D). Proteins categorized as "Extracellular Space" in either "neuron only" or "both" occupy a very small percentage (2%), in line with the predominant

cellular protein content in these categories. While extracellular proteins in “brain only” category show a much larger percentage (13%), consistent with the fact that the brain tissue contains multiple cell types as well as inter-cellular connections such as myelin sheath and extracellular matrices, and is therefore more heterogeneous. Because we analyzed cytosolic fractions, proteins annotated as “nuclear” occupied a relatively small fraction in all three categories. Interestingly, a fair amount of plasma membrane proteins, ranging from 10% to 22%, were also identified, even without the enrichment of membranes.

Because an equal amount of ^{15}N brain homogenates were spiked into each sample, the ratio between ^{14}N and ^{15}N peptides can be used to represent the relative protein expression ratio between samples, as long as the neuronal protein is also identified in the adult brain^{8,9}. From two replicate MudPIT analyses of one group of neuronal samples, we quantified 4348 proteins in control neurons and 4049 proteins in PCP treated neurons, with a significant degree (3217) of overlap between these sets (Figure 2A). For these proteins with overlapping quantifications, we were able to compare protein expression levels between the two groups of neurons. To assess the precision of the quantification, we plotted the correlation of over 3000 protein ratios between control neurons and PCP treated neurons, as well as the correlation of protein ratios between the two technical runs of the same sample (Figure 2B). In either cases, the Pearson's correlation coefficient is greater than or equal to 0.97, with P values less than 0.0001, indicating very good correlations. The good correlation of protein ratios between the two technical runs demonstrates the accuracy of this quantitative method, while the excellent correlation of protein ratios between the control and PCP treated neurons supports the idea that a 15 minute treatment of cortical neurons with PCP renders no changes in overall protein levels. Because our goal is to identify rapid changes in protein phosphorylation, no change in protein expression levels can serve as a reference point for a regulated phosphorylation change.

The ratio-over-ratio between PCP treated and control neurons represents the protein expression ratio between the two groups of neurons. The log transformed distribution of over 3000 protein ratios follows a Gaussian distribution, with the coefficient of determination (R^2), a measurement of goodness of fit, greater than 0.97 in both replicates (Figure 2C). We also performed SIL in cells experiment in which neurons were grown in either light or heavy media, and treated similarly with either control or PCP. Our data show that in primary cultured neurons, quantification by SILAM is superior to the one based on SIL. Although the protein ratios generated from SIL also fit to a Gaussian distribution but with a lower R value and the distribution of the ratios is much broader indicating more variation in the values, which indicates that the quantification is less accurate (Figure 2D).

Identification of phosphoproteome and bioinformatic analysis of phosphorylation sites

We enriched phosphopeptides using a combined HILIC-IMAC approach¹⁹ from total rat cortical neuronal cell lysates after mixing with the SILAM rat brain homogenates. We used a 10 ppm precursor ion mass tolerance for all phosphorylated peptides and a false discovery rate at the peptide level lower than 1%. From two technical replicates of the phosphopeptide enriched neuronal lysates, we identified between 1525 and 2936 ^{14}N unique phosphopeptides with a phosphopeptide enrichment ratio between 51% and 69%. The number of corresponding unique phosphoproteins identified ranged from 647 to 846 (Figure 3A). Searching by ^{15}N in the same MudPIT analysis, we identified 331 to 728 phosphopeptides corresponding to 178 to 344 ^{15}N phosphoproteins (Figure 3A). The phosphopeptide enrichment ratios also ranged between 49% and 65%. The similar enrichment ratios between the cultured neurons and the brain tissue suggest that our HILIC-IMAC approach to enrich phosphopeptides is not biased toward either homogeneous neuronal cells or highly complex brain tissue. In either the neurons or the brain tissue, the phosphorylation events at the three residues are 2–3% at tyrosine, 15–17% at threonine, and

81–82% at serine (Figure 3B). This distribution is consistent with all previous large-scale studies to identify phosphorylation sites^{18, 35}.

We performed a replicate biological experiment using the same HILIC-IMAC method to enrich for phosphopeptides and pulled all phosphopeptide identifications into three groups: control neurons, PCP neurons, and the brain tissue. The total numbers of phosphopeptide (non-unique) identifications in these groups are 15247, 14139, and 6948, respectively; and the total numbers of phosphorylation sites are 18239, 16824, and 7785, respectively. Using a phosphorylation site localization algorithm based on binomial probability²⁹, we assigned a localization probability score for each site, and found that we could obtain statistically significant scores ($P < 0.05$) for 2/3 of the sites (Figure 3C). After removing redundancy we confidently identified 3370, 2762 and 1408 unique phosphorylation sites from the three respective groups (Figure 3C) with the union totaling 4647 sites. All phosphopeptides and their site localization results are listed in Table S7.

To estimate the abundance of phosphorylation sites within each sample we used spectral counting to represent the number of times a peptide is analyzed by the mass spectrometer. Spectral counts correlate with protein abundance³⁶, and have also been applied to approximate the abundance of phosphorylation sites in mouse tissues³⁷. The 4647 sites that were confidently localized from all three sample categories were analyzed by hierarchical clustering with spectral counting as the cluster metric, and presented as a heat map in Figure 3D and Table S1. Not surprisingly, the distribution pattern of phosphorylation site abundance between control neurons and PCP treated neurons shows far more similarity than between neurons and the brain, indicating that the brain has distinct phosphorylation patterns compared to dissociated neurons.

To extract the amino acid sequence motifs flanking the phosphorylation sites that are recognized by kinases, we applied a pattern recognition algorithm, Motif-X³⁰, that has been successfully applied in a number of large scale phosphorylation studies³⁷. In this study, only the confidently localized phosphorylation sites were scanned by Motif-X. In addition to extracting common motifs, Motif-X also computes a fold increase of each motif by comparing the total number of peptides for the same motifs in the sample to that found in the background database. We use this fold increase value as an intensity value to represent all the motifs identified in the three samples, analyzed by hierarchical clustering and plotted in a heatmap (Figure 3E and Table S2). Overall, the phosphorylation site motifs of the two groups of neurons clustered together by similarity, while the motifs from the brain appear to be more distant. Proline-directed and acidophilic kinase motifs comprise the vast majority of the motifs identified (>80%) in the neurons, with only a few motifs being basophilic, similar to previous reports³⁷. We found many more motifs in the neurons than in the brain, presumably due to many more phosphopeptide identifications in neurons. Only one motif that encompasses the KSP residue is exclusively identified in the brain, and this motif was also identified in our previous studies in adult brain⁷.

Quantification of the phosphoproteome in primary neurons

The two technical replicates allow us to assess the reproducibility of the quantitative phosphopeptide analysis. The correlation between the two control ratios or the two PCP ratios shows a square of Pearson's correlation coefficient greater than 0.85. In contrast, the correlation between the control ratios against the PCP ratios shows a square of Pearson's correlation coefficient of 0.79 (Figure 4A) and 0.74 (Figure S2), a much poorer correlation. These results suggest that there are differences in phosphorylation between these sets that are a result of PCP treatment. Putting together two biological replicate analyses of control and PCP treated neurons, we quantified 2965 phosphopeptides from control neurons and 2958 phosphopeptides from PCP treated neurons. The overlapping phosphopeptides (1556)

allowed us to quantify the relative phosphorylation events between the control and PCP treated neurons (Figure 4B and Table S3). The distribution of the phosphopeptide ratios between PCP and control neurons largely follows a Gaussian distribution, with the coefficient of Gaussian fit (R^2) equal to 0.96 (Figure 4C). To obtain significantly changed phosphorylation events, we took the ratios between PCP and control samples from the two biological replicates, and then performed an ANOVA test on the peptides that were quantified in both replicates. For the peptides that were quantified in one experiment, we did the outlier test to find the largest changes. This leads to the identification of 109 phosphopeptides that are either up or down in phosphorylation levels, with the majority (84 phosphopeptides) down.

We used a targeted multiple reaction ion monitoring (MRM) strategy³⁸ to validate ten selected phosphopeptides that were differentially phosphorylated from the pool of 109 changed phosphopeptides. Heavy ($^{13}\text{C}^{15}\text{N}$) arginine or lysine-containing phosphopeptides were used as spike-in internal standards to obtain the absolute quantity of the neuronal lysates. Lysates from neurons treated with PCP or left untreated were spiked with known amount of the heavy phosphopeptide standards and were quantified using MRM. Four out of the ten peptides didn't result in quantifiable MRM peaks. For the remaining six phosphopeptides, the ratios calculated by MRM are consistent with the results from SILAM approach (Table S4). As an example, the MRM chromatogram of a phosphopeptide derived from a membrane channel protein, GABRA3, showed an increase in absolute amount of phosphorylation after PCP treatment (Figure 5, A–E), consistent with the trend observed in the SILAM experiments.

The proteins derived from these phosphopeptides were categorized using Gene Ontology and are shown in Figure 4D. Except for proteins with unknown function, a large percentage of these proteins are related to cellular structure (18%), signal transduction (15%), and nucleotide binding (10%). Other proteins were grouped into a diverse set of functions, including transcription, transport, synaptic transmission, neurogenesis, translation, and ion channels. These proteins were further analyzed by the Ingenuity pathway analysis software. Ninety nine out of the 109 proteins with changes in phosphorylation were mapped into Ingenuity knowledge base. Fisher's exact test was then applied to test the significance of the association of the phosphoproteins in different signaling pathways. The canonical signal transduction pathways significantly enriched ($P < 0.05$) are shown in Figure 4F. Almost all of the over-represented pathways are intrinsically associated with neuronal signaling, including amyloid processing, CREB signaling, synaptic long-term potentiation, and CDK5 signaling. Remarkably, the neuregulin signaling pathway, which acts through the ERBB family of receptor tyrosine kinases to induce the growth and differentiation of a large number of ectoderm-derived cells, including neurons, also showed significant over-representation. In contrast, the significantly over-represented categories from a subset of unchanged phosphoproteins that were randomly selected to have the same number of proteins as the changed ones show no trend toward signaling pathways enriched in the neuronal system; they are enriched in such fundamental cellular processes as amino acid metabolism and immune response (Figure S3). On the other hand, even though we detect strong reduction of ERK1/2 phosphorylation, ERK/MAPK signaling did not pass the threshold for over-representation.

The glutamate mediated signal transduction pathway initiating from presynaptic glutamate release to the activation of various type of glutamate receptors, leading to the activation of multiple intracellular signaling nodes are presented in Figure 4E. Three important signaling nodes in this network, PKA, PKC and Ras showed a decrease in phosphorylation (Green), only mGLUR showed an increase in phosphorylation. This is consistent with the overall repression of phosphorylation and the activity of the glutamatergic system mediated by PCP

inhibition. In addition to a specific enrichment of these 99 proteins with neuronal functions, 38 are related to neurological disease with 21 related to psychological disorders. Specifically, 9 proteins are associated with schizophrenia, 7 with depressive disorder, 15 with mood disorder, and 3 with psychosis (Table S6). This result suggests that for many proteins previously found to associate with neurological disorders mainly through genetic studies, changes in proteins phosphorylation may contribute to the underlying mechanisms of these diseases.

PCP modulates inhibitory neural transmission in primary cortical neurons

Changes in phosphorylation of membrane ion channels have been shown to result in changes in their channel properties and neuronal excitability²¹, we went on to test if, after prolonged inhibition, the initial changes in phosphorylation events can lead to protein level changes in several key molecules forming the excitatory and inhibitory synapses. The Shank proteins are scaffold molecules that support the morphological integrity of postsynaptic density, the key structure of the excitatory synapse. GABA-A receptors are ligand-gated chloride channels that form the basis of the inhibitory synapse. Membrane-enriched lysates of cortical neurons treated with either PCP or MK801 were analyzed by immunoblotting with antibodies against several synaptic proteins, shown in Figure 6A and 6B. MK801 is another non-competitive NMDA receptor antagonist that has similar pharmacological effects as PCP. Neurons treated with PCP or MK801 for 15 minutes show no change in any of the proteins examined. Three hours of treatment caused down-regulation in GABRA1 and GABRA3 receptors, and dramatic up-regulation in Shank proteins in the membrane fraction. No change in NR2B subunit of the NMDA receptor is found. The enrichment of synaptic proteins is demonstrated by comparing the total neuronal homogenate with the membrane fraction using GABRA1 receptor (Figure 6A). One potential kinase that can recognize the proline-directed phosphorylation site in GABRA3 (S434, Figure 5F) is CKD5, whose signaling node was found to have changed in phosphorylation (Figure 4F). We therefore examined the level of membrane-bound CDK5. Treatment with PCP or MK801 for either 15 minutes or 3 hours did not result in appreciable change in CDK5, suggesting the level of CDK5 might not be responsible for the changes in GABRA3 phosphorylation.

To test the hypothesis that phencyclidine (PCP) may affect the function of native GABA receptors, we performed electrophysiological recordings of GABAergic synaptic transmission in cultured cortical neurons. At 14–16 days in vitro, these neurons form functional excitatory and inhibitory synapses and exhibit robust postsynaptic currents in response to action potentials. In agreement with previously published observations³⁹, we found that in the presence of excitatory AMPA and NMDA receptors blockers CNQX and AP-5, single action potentials elicited typical GABAergic inhibitory postsynaptic currents (eIPSCs). Strikingly, incubation of neurons with 50 μ M PCP after 1 or 3 hours resulted in a dramatic reduction of eIPSC amplitudes (Figure 6C, 6D) without affecting the eIPSC frequencies (Figure S4). The wash-out of PCP fully reversed this phenotype suggesting that PCP does not significantly change the densities of inhibitory synapses. To rule out the possibility that PCP affects the probability of presynaptic vesicular transmitter release, we plotted the ratios of IPSCs recorded from control or PCP-treated neurons stimulated by pairs of action potentials (Figure 6E). No significant difference in paired pulse ratios was found suggesting that PCP-induced reduction in inhibitory synaptic strength is likely to be due to changes in density or properties of postsynaptic GABA receptors. This result is in line with the biochemistry data shown in Figure 6A, and suggests that PCP-mediated neuronal signaling may act through a previously unidentified mechanism to reduce the membrane active GABA receptors.

Discussion

To our knowledge, this study provides the first large scale quantitative view of the phosphoproteome in primary neurons after perturbation of neuronal activity. We expanded the applicability of stable isotope labeled mammals which we developed previously⁹, to make use of the completely ¹⁵N labeled rat brain lysate as a common internal standard. This allowed for the relative quantification of neuronal proteome and phosphoproteome, bypassing the difficulty of labeling postmitotic neurons. Using this approach, we obtained accurate information of the relative levels between experimental conditions of ¾ of the identified neuronal proteome and phosphoproteome. We used a serial fractionation and enrichment strategy to enrich phosphopeptides. Through bioinformatics analysis of the phosphorylation sites, we uncovered a previously unrecognized downstream effector of phencyclidine. Our results show that short-term PCP treatment of cortical neurons led to a decrease in ERK phosphorylation and changes in phosphorylation in 7% of quantified phosphopeptides. Multiple proteins with altered phosphorylation levels are involved in modulation of synaptic strength and implicated in neuropsychiatric disorders. Hours of PCP treatment results in protein level changes in some receptors from both excitatory and inhibitory neurotransmitter systems. Specifically, PCP changes the electrophysiological properties of inhibitory neural transmission.

Our previous work based on stable isotope labeling in cultured neurons indicates that different proteins incorporate heavy isotopes with different rates³, presumably as a result of the balance between protein synthesis and stability. While protein synthesis on a global scale is largely determined by cell growth and division in mitotic cells, the stability of proteins is correlated with protein sequence motif and N-terminal amino acid residue⁴⁰. Therefore, assuming complete isotope labeling in all proteins in primary neurons will lead to quantitative errors. Moreover, in studies that require harvesting cultured neurons in very early developmental stages, heavy isotope labeling is not applicable. As shown in this study SILAM labeled brain tissue can be used as a common internal standard to effectively overcome this problem. In addition, because each biological condition is analyzed independently with the common internal standard (e.g. ratio of ratios), this methodology is well suited to experiments with multiple conditions. Based on our results, we also believe that different SILAM tissues can be used as common internal standards to quantify a variety of primary cells in which heavy isotope labeling cannot reach completion in cell culture conditions.

Multiple factors can affect the reproducibility of identifying and quantifying the same protein or phosphoprotein in different runs. Abundance of the protein in the complex mixture, ionization efficiency of peptides with different amino acid sequences, and the stochastic sampling process of the mass spectrometry data acquisition routines all contribute to the missing data in one run versus the other^{36, 41}. With the advancement of mass spectrometry technology – where continuous improvement in data acquisition speed is achieved while maintaining high resolution and mass accuracy⁴², this problem can be greatly minimized but not completely overcome. Therefore, in our quantification approach, obtaining quantitative information for proteins that do not overlap between experimental conditions is challenging, a subset of which can be potentially treated as an all-or-none situation in which there were dramatic differences in protein expression or phosphorylation events between different biological conditions. The development of more advanced software algorithms to accurately capture these subset proteins is needed to maximize the coverage of proteomic data.

Because of the low stoichiometry, it is necessary to enrich phosphorylated proteins or peptides before mass spectrometry analysis in order to identify a large number of

phosphorylation events. We combined fractionation with HILIC followed by phosphopeptide enrichment with Fe³⁺-based IMAC, and multi-stage activation when acquiring tandem mass spectra and thus maximized our identification of phosphopeptides. Although in most experiments we started with one milligram of total protein as an input for the entire procedure, we also found that doubling the input protein amount does not necessarily improve our phosphopeptide identifications. This is probably due to the capacity limitation of the HILIC column²³ because another type of fractionation, strong-cation exchange (SCX), has a capacity extending to tens of milligrams¹⁶.

Our quantitative analysis found that 7% of the quantified phosphopeptides significantly changed. Consistent with the inhibitory effect exerted by PCP to excitatory neural transmitter receptors, a majority of these changes are reduction in phosphorylation levels. Surprisingly, we found increased phosphorylation of a well-documented phosphorylation site (S433) of GABRA⁴³. Phencyclidine and analogues are known to influence GABA-mediated inhibitory neural transmission, by inhibiting NMDA stimulated GABA release⁴⁴, or by influencing GABA(A) receptor subunit gene expression⁴⁵. Remarkably, a GABRA3 knockout mouse showed dramatic attenuation in prepulse inhibition of the acoustic startle response, indicating a severe deficit in sensorimotor gating, a common condition in schizophrenia patients⁴⁶. Here we observed that treatment of cortical neurons with PCP results in reduction of membrane GABRA3 receptor, this reduction may partially account for PCP mediated prepulse inhibition in rodents. Based on previous findings that phosphorylation of ion channels leads to conformational change of the channel pore that alter gating properties²¹, that phosphorylation can also lead to protein trafficking-dependent changes in surface expression of ion channels⁴⁷, we postulate that phosphorylation changes in GABRA3 may be the initial signaling event that leads to changes in its membrane levels and eventually changes in inhibitory postsynaptic currents, as shown in Figure 6. Identifying the kinases and phosphatases responsible for the phosphorylation on the GABRA3 site will be a critical next step further dissecting the signaling pathway initiated by PCP.

The 9 phosphoproteins in our data set that have been associated with schizophrenia provide further insights into a mechanistic understanding of schizophrenia. In addition to the documented findings in GABRA3, messenger RNAs for a growth associated protein (GAP43) and a neuronal navigator protein (NAV1) were reduced in the dorsolateral prefrontal cortex of schizophrenia patients, suggesting the reduction of plasticity in synaptic terminals⁴⁸. On the other hand, the mRNA of a neurite outgrowth inhibitory protein Nogo (RTNs) is elevated in schizophrenia cortex⁴⁹. Genetic studies have provided evidence that there is an alteration in the allele frequency distribution of GRM5 (a type of metabotropic glutamate receptor) gene in schizophrenia patients compared with control population⁵⁰. Given the importance of protein phosphorylation in regulating protein-protein interaction, it's tempting to speculate that changes in phosphorylation of these proteins, so far known to associate with schizophrenia mostly through genetic studies, may change their association with other proteins and thereby disrupt their functionalities. Together, the results of this study provide valuable insights into the dynamics of molecular networks already implicated in schizophrenia, and suggest that dynamic signaling events other than protein expression differences may be related to the etiology of schizophrenia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. SILAM method to quantify proteome in primary neurons. (A) Overview of the method. (B) Western analysis indicates a decrease in ERK phosphorylation after 15 minutes PCP treatment in primary cortical neurons. (C) The number of identified proteins in neurons, brain, or both. The overlap between the neurons and the brain is 52%. (D) Gene Ontology analysis of the identified proteins shows the coverage of all cellular fractions.

Figure 2.

Accuracy of quantification. (A) Upper panel: The number of quantified proteins and peptides in either control or PCP treated neuronal samples. Lower panel: Venn diagram showing the large overlap of quantified proteins between control and PCP treated neurons. (B) Pearson's correlation of $^{14}\text{N}/^{15}\text{N}$ protein ratios between two technical replicate runs of the same control sample (left), and of $^{14}\text{N}/^{15}\text{N}$ protein ratios between the control and PCP treated neurons (right) shows good precision and reproducibility of quantification. (C) The distributions of protein ratios (obtained by ratio-over-ratio calculation) between the control and PCP treated neurons acquired by SILAM follow normal distribution with very narrow deviation. (D) The distributions of protein ratios between the control and PCP treated neurons acquired by SIL experiments also follow normal distribution but the deviation is large.

Figure 3.

Identification of phosphoproteome. (A) Phosphopeptides and phosphoproteins contributed from neurons can be identified by ^{14}N searches, while those from the brain tissue can be identified by ^{15}N searches. The enrichment ratios of phosphopeptides using the HILIC-IMAC approach were shown. (B) The distribution of the three observed phosphorylated amino acid residues from either the neurons or the brain are the same. (C) Frequency distribution of phosphorylation sites at different samples. (D) Motif extracted from the localized phosphorylation sites. The intensity of each motif represents the fold increases of each motif as compared with the background database. (E) Hierarchical clustering of phosphorylation sites in control neurons, PCP treated neurons, and brain. The intensity of each site is represented as spectral counts.

Figure 4.

Quantification of phosphoproteome. (A) Pearson's correlation of $^{14}\text{N}/^{15}\text{N}$ phosphopeptide ratios between two technical replicate runs of the same control sample (left), and of $^{14}\text{N}/^{15}\text{N}$ phosphopeptide ratios between the two technical replicate runs of the same PCP treated sample (middle) shows good correlation; but the correlation deteriorates between the control and PCP treated samples. (B) Venn's diagram shows that about two-thirds of the quantified phosphopeptides between the control and PCP treated samples overlap, allowing the calculation of ratio-over-ratio. (C) Histogram of phosphopeptide ratios between PCP treated and control neurons, an expression ratio calculated as ratio-over-ratio, follows normal distribution. (D) Gene Ontology analysis of changed phosphoproteins indicates that these proteins span a wide range of cellular functions. A large portion is involved in signaling transduction and neuronal function. (E) Protein network analysis displays the glutamate mediated signaling with changed phosphoprotein labeled with green (down) or red (up). (F) Fisher's exact test using hypergeometric distribution shows that proteins with changed phosphorylation events are enriched in multiple signaling pathways involve in neuronal signaling. The numbers in the graph indicates the total number of proteins in a given pathway in the Ingenuity knowledgebase.

Figure 5.

SRM validation of the GABRA3 phosphopeptide quantified by SILAM. Spiked-in synthetic heavy peptide with known concentration was used to absolutely quantify the phosphopeptide. Total ion current of the endogenous, light version of the peptide and the heavy peptide (A, B), as well as the respective SRM transition ion spectra of the two peptides (C, D), are shown. (A, C) are the spectra generated from control samples and (B, D) are spectra generated from PCP-treated samples. (E) Student's T-test shows the significant difference in the absolute levels of the GABRA3 phosphopeptide between control and PCP-treated neurons from three batches of cultures. (F) Tandem mass spectrum of the phosphopeptide supports the patterns of the fragmentations used in SRM experiments.

Figure 6.

PCP causes downregulation in GABA mediated inhibitory neural transmission. (A) Western blot analysis of synaptic membrane fractions of cortical neurons cultured at 14 days in vitro. Fifteen minutes treatment with either 50 μ M of PCP or 40 μ M of MK801 exerted no effect on all proteins examined. In contrast, 3 hours treatment of the same agents caused down-regulation of membrane bound GABA-A receptor subunits, and up-regulation of Shank. GABRA1 is used to show the enrichment of synaptic membrane proteins. (B) Bar graph representation of relative protein levels observed from Western blot normalized to loading control after background subtraction. Statistical analysis using Student's T-test shows the significance of the changed proteins. (C) and (D), inhibitory GABAergic postsynaptic currents (IPSCs) were monitored from cortical neurons cultured in vitro for 14–16 days. IPSCs were recorded in a whole cell mode in the presence of excitatory AMPA and NMDA receptors blockers CNQX (20 μ M) and AP-5 (50 μ M). IPSCs were triggered by single action potentials from control neurons, PCP-treated neurons, and PCP-treated neurons that were returned to normal culture medium prior to electrophysiological experiments. Typical IPSC traces (C) and the averaged amplitudes of IPSCs (D) recorded in four independent experiments are shown. The total numbers of analyzed neurons are indicated on each bar. (E) Paired-pulse stimulation shows no change in all treatment paradigms. IPSCs were triggered by two action potentials applied at different intervals. The ratios of IPSC amplitudes were plotted as a function of inter stimulus interval