

Supplementary Materials for Neurotransmitters Drive Combinatorial Multistate Postsynaptic Density Networks

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Materials and Methods Supplemental Data

Peptide Array Phosphorylation Assays—Jerini Phosphosite detector™ peptide arrays (Jerini Peptide Technologies, GmbH) were used to analyze 600 peptide sequences corresponding to 91 PSD proteins with 25 protein kinases. 15-amino acid-long peptides that encompassed the selected sites were synthesized on cellulose membranes in a parallel manner using SPOT technology, deposited to glass slides, and covalently immobilized to the glass slide surface. Each peptide was present in triplicate on the chip, and seven full-length proteins capable of being phosphorylated were also present. Negative control peptides for each phosphorylation site were included, replacing serine, threonine, or tyrosine with alanine, valine or phenylalanine, respectively. Consensus phosphorylation sequences for the kinases analyzed were also attached. Peptide arrays were sealed with Gene-Frame™ incubation chambers (Abgene house, Surrey, UK), and the chambers were filled with 330 μ l of kinase buffer, 20 mM MOPS, pH 7.2, 25 mM β -glycerol phosphate, 5mM EGTA, 1mM sodium orthovanadate, 1mM DTT for PKA (with 2 μ M cAMP), Cdk5 /p35, ERK1, ERK2, p38 α , MEK1, Casein Kinase 2, Casein Kinase 1, Raf-1, Rsk2, ROCK-II, TBK1, GSK3 β , PKC ζ , Fes, Pyk2, Src, p70 S6 Kinase and Akt1, 20 mM MOPS, pH 7.2, 25 mM β -glycerol phosphate, 1mM sodium orthovanadate, 1mM DTT, 100 μ M CaCl₂ for CaMKII (40 μ g/ml Calmodulin) and PKC α (1mg/ml phosphatidylserine, 0.1mg/ml Diacylglycerol), 50 mM Tris-HCl, pH 7.5, 1mM EGTA, 1% (v/v) 2-mercaptoethanol for PDK-1, JNK3 and Fyn, 20 mM HEPES, 0.03% Triton X-100, 1mg/ml phosphatidylserine, 0.1mg/ml Diacylglycerol for PKC δ .

Each kinase was assayed to determine the concentration needed to achieve optimal signal/noise ratio using test protein chips. Kinase activity was tested against proteins and peptide controls for each kinase and assayed by Western blot and peptide arrays. 500U/mg (Activity), (1 μ g-4 μ g) of protein kinases were included in the corresponding buffer.

Reactions were initiated by the addition of ATP/MgCl₂, 100 μ M ATP, 10 μ Ci [γ -³²P] ATP (Amersham Biosciences) 15mM MgCl₂ final concentration. After a 40 minutes at 32 °C incubation, the peptide microarrays were washed six times, alternating between 0.1 M phosphoric acid and distilled water. γ -³²P incorporation in the immobilized peptide spots was detected on a Typhoon 8600 PhosphorImager (50- μ m resolution; Amersham Biosciences). Image analysis and signal quantification was carried out using ImageQuant TL (Amersham Biosciences), and positive signals were defined after background subtraction.

Preparation of hippocampal slices

Experiments were performed on hippocampal slices obtained from 12 to 14 weeks old 129S5/SvEvBrd mice bred at the Wellcome Trust Sanger Institute essentially as described (58). Animals were sacrificed by cervical dislocation in accordance with Schedule 1 to the U.K. Animals (Scientific Procedures) Act 1986. Whole brain was immediately transferred to a beaker containing ice-cold

solution of the following composition (in mM): sucrose 110, NaCl 60, NaHCO₃ 28, NaH₂PO₄ 1.25, KCl 3, MgSO₄ 7, CaCl₂ 0.5, glucose 5, sodium ascorbate 0.6, phenol red 0.015. Prior to use, this solution was thoroughly saturated with a gas mixture of 95%O₂/5%CO₂ pH 7.25-7.35. Brain was allowed to chill for 2-3 min and then it was trimmed and mounted on the stage of a Vibroslice MA752 (Campden Instruments, Loughborough, UK). Slices were cut at 350 μm, placed into a well of the slice chamber (Fine Science Tools, Foster City, CA), and kept submerged under the constant flow (2 ml/min) of fresh artificial cerebrospinal fluid (ACSF) containing (mM): NaCl 124, NaHCO₃ 25, NaH₂PO₄ 1, KCl 4.4, MgSO₄ 1.2, CaCl₂ 2, glucose 10, phenol red 0.015. When slices were being transferred to the slice chamber, ACSF temperature was maintained at 23 °C and then gradually warmed to 30 °C for the rest of the incubation period. Before the start of biochemical stimulations, slices rested in these conditions for at least two hours after the last slice was cut.

Biochemical stimulation

Experiments were usually performed on slices from four animals simultaneously and slices from the same animal were stimulated or left untreated (DMSO control). Slices were incubated in the “treated” chamber with the following solutions 20μM NMDA (3 min), 50μM forskolin (15 min), Wortmannin 200nm (40 min), PdBu 10μM (15 min), SB415286 10μM (90 min), KN-62 10μM (30 min). All drugs were from Tocris Cooksoon Ltd (Bristol, UK.).

To facilitate solution exchange in the slice chamber well, the speed of solution flow was increased to 5-7 ml/min for 3 min during the start of incubation with drugs and upon their wash-out.

PSD-enriched fractions were prepared using a 3 step protocol. Hippocampal slices were homogenized in 10mM HEPES buffer pH 7.4, containing 2mM EDTA, 5mM Sodium Orthovanadate, 30mM NaF, 20mM β-glycerol phosphate and Roche Complete as protease inhibitor cocktail. Homogenates were spun, for 4 min at 500g supernatant collected, and centrifuged at 10,000g, and the membrane fraction solubilized in HEPES 50mM pH 7.4, containing 2mM EGTA, 2mM EDTA, 30mM NaF, 5mM Sodium Orthovanadate, 20mM β-glycerol phosphate, Roche Complete and 1% Triton X-100. Solubilized membranes were centrifuged at 30,000 RPM in a Beckman Optima Max rotor MLA-130 for 40 minutes, pellet was collected and solubilized in 50mM Tris pH 9, 30mM NaF, 5mM Sodium Orthovanadate, 20mM β-glycerol phosphate, Roche Complete and 1% Sodium Deoxycholate (DOC).

Standard techniques were used for Western Blot analysis of the samples. For primary antibody information see Supplementary Table 17.

Slice stimulation and phosphopeptide isolation:

Hippocampal slices from the same animals were treated and non-treated, with NMDA in 6 independent assays, for a total of 24 mice.

The Triton X-100 insoluble fraction was isolated from NMDA-treated (20 mM NMDA for 3 min) and control mouse hippocampal slices. This fraction (3 mg) was solubilized in 1 % deoxycholate, treated with TCEP, and digested with trypsin for 4 hours. Desalted peptides were methyl-esterified for 2 hours phosphopeptides were isolated using gallium IMAC as described previously (1).

Statistical overlap between sets of molecules

The statistical significance of an overlap between two sets of molecules was calculated using the method of Pocklington *et al.*(2). Suppose that out of N molecules, n_a and n_b belong to sets {a} and {b} respectively. If a and b are randomly distributed throughout N, the probability of finding n_{ab} molecules belonging to both sets is given by the function:

$$h(n_{ab}, n_a, N, n_b) = n_a!(N-n_a)!n_b!(N-n_b)!/[N!(n_a-n_{ab})!n_{ab}!(N-n_a-n_b+n_{ab})!(n_b-n_{ab})!].$$

Given the actual number of molecules μ_{ab} belonging to both annotations, we estimate its significance by calculating the probability $P(\mu_{ab})$ of an overlap as or less likely under the random distribution:

$$P(\mu_{ab}) = \sum h(n_{ab}, n_a, N, n_b) : h(n_{ab}, n_a, N, n_b) \leq h(\mu_{ab}, n_a, N, n_b)$$

This test was used in the analysis of the following datasets:

Phosphorylation of substrate classes by kinase classes – N = total number of multiply phosphorylated array sites (95), {a} = sites present in substrates from functional class a, {b} = sites primarily phosphorylated by kinase class b.

Phosphorylation of sites within functional motifs by kinase classes – N = total number of phosphorylated array sites found within functional motifs, {a} = sites present in motif a, {b} = sites primarily phosphorylated by kinase class b.

Phosphoproteome network clustering

For the purposes of clustering, kinase-kinase and kinase substrate phosphorylation interactions were represented as an undirected graph. Kinases and substrates were represented as nodes and interactions as links between pairs of nodes. This network was clustered using the algorithm of Newman & Girvan (3), followed by local optimisation of the resulting clusters to maximise the modularity score (3)

Definitions of peptide phosphorylation status in NMDA stimulation experiments

Peptides were grouped into 10 classes as follows.

Class A: Peptides that could be considered as **Phosphorylated** with NMDA treatment

- i. Peptide present in treated (NMDA) samples only (not in untreated).
- ii. Ratio of peptide intensity in treated relative to untreated greater than 1.5.
- iii. Intensity ratio greater than 1 in one experiment/peptide phosphorylation state, NMDA enriched (class A: i or ii) in another.
- iv. Increase in number of phosphates with NMDA (in both experiments) - different forms of this peptide (e.g. lower number of phosphates) observed in class B or C.

Class B: Peptides that could be considered as **Dephosphorylated** with NMDA treatment

- i. Peptide present in untreated samples only (not in NMDA).
- ii. Ratio of peptide intensity in treated relative to untreated less than 0.5.
- iii. Intensity ratio less than 1 in one experiment/peptide phosphorylation state, Control enriched (class B: i or ii) in another.
- iv. Decrease in number of phosphates with NMDA (in both experiments) - different forms of this peptide (e.g. lower number of phosphates) observed in class A or C.

Class C: Peptides whose phosphorylation state could be considered as **Unchanged** with NMDA treatment

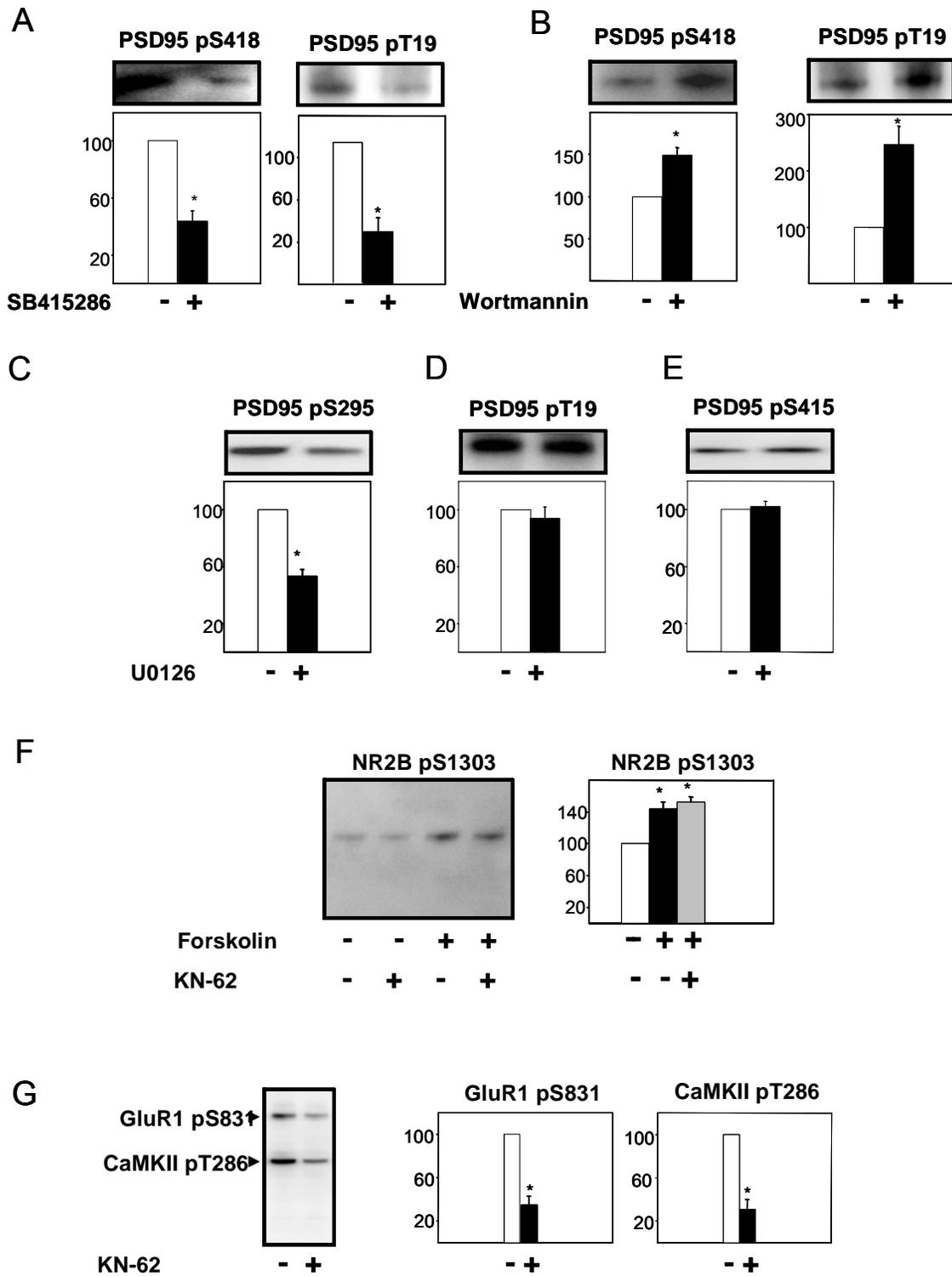
Ratio of peptide intensity in treated relative to untreated lies between 0.5 and 1.5 (in all experiments/phosphorylation states in which the peptide is found).

Class D: Unclear/Inconsistent

Multiple peptides, assigned to different classes (A B or C), or single peptide assigned to different classes in different experiments.

Analysis of modulated peptides was carried out using classes A and B (i-iv). Inclusion/exclusion of subclass (iv) did not significantly affect the results.

Supplementary fig. 1. In vivo validation of peptide arrays.



The activity of specific kinases in hippocampal slices was regulated pharmacologically and phosphorylation of substrates assayed using immunoblotting of protein extracts. Four phosphorylation sites in PSD-95 (Thr¹⁹, Ser²⁹⁵, Ser⁴¹⁸ and Ser⁴¹⁵) were monitored (A-E). Consistent with the in vitro data showing Ser⁴¹⁸ as a GSK3 β substrate, treatment of hippocampal slices with the GSK3 inhibitor SB415286 (4) showed a 56% \pm 7 (p<0.005) decrease in PSD95 Ser⁴¹⁸ phosphorylation and a 67% \pm 9 (p<0.005) decrease in PSD95 Thr¹⁹ phosphorylation (A). Moreover, activation of GSK3 pathway by blocking the phosphoinositide-Akt-dependent inhibition of GSK3 with wortmannin (5) led to an increase (149% \pm 9p<0.01) in PSD95 Ser⁴¹⁸ phosphorylation and an increase (277 \pm 14 p<0.005) in PSD95 Thr¹⁹ phosphorylation (B). Consistent with in vitro data, PSD95 Ser²⁹⁵ was modulated by the ERK1/2 pathway in vivo: treatment of hippocampal slices with the specific MEK inhibitor U0126 led to a 47% \pm 5 (p<0.01) decrease in Ser²⁹⁵ phosphorylation (C). The same treatment had no effect on PSD95 Thr¹⁹, which was phosphorylated in vitro by a different proline-directed kinase (Cdk5) (6) (D), or on the non-proline-directed site PSD95 Ser⁴¹⁵ (phosphorylated in vitro by TBK1) (E). As expected from published studies (7), we found that the C terminus phosphorylation site in NR2B (7) Ser¹³⁰³ was phosphorylated by CaMKII in peptide arrays experiments. However, we found this site was also phosphorylated by PKA in vitro and we tested if the convergence between CaMKII and PKA onto NR2B Ser¹³⁰³ occurred in vivo. Consistent with PKA phosphorylation, treatment of hippocampal slices with forskolin (FSK) resulted in an increase in the NR2B Ser¹³⁰³ phosphorylation of 144% \pm 8 (p<0.001) (F). To exclude the possibility of an indirect role of CaMKII activation by FSK, we performed the assay in the presence of the CaMKII inhibitor KN-62, with no effect on the FSK-mediated NR2B Ser¹³⁰³ phosphorylation. Inhibition of CaMKII activity after KN-62 treatment was demonstrated by decreased CaMKII substrate (GluR1 Ser⁸³¹) phosphorylation and reduced kinase autophosphorylation (CaMKII Thr²⁸⁶) (G). Total protein content remained unchanged in all the conditions tested.

A. Inhibition of GSK3 β activity with SB415286 (10 μ M, 90 min) decreases PSD95 Ser⁴¹⁸ and Thr¹⁹ phosphorylation (p<0.005).

B. Release of the phosphoinositide-Akt-dependent inhibition of GSK3 β and concomitant increase in PSD95 Ser⁴¹⁸ phosphorylation with wortmannin (200 nM, 90 min) (p<0.01), PSD95 Thr¹⁹ (p<0.005).

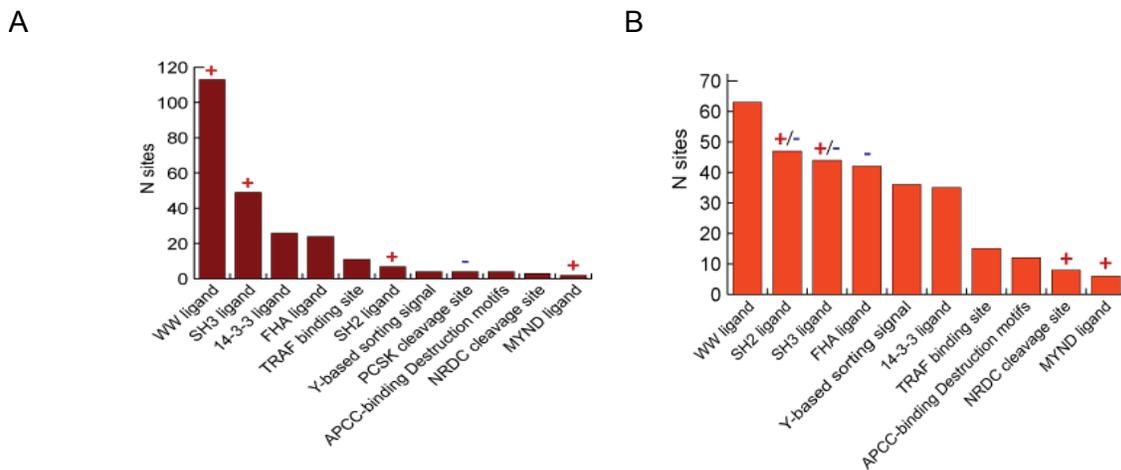
C. The modulation of PSD95 Ser²⁹⁵ phosphorylation by ERK1/2 pathway with the specific MEK inhibitor, U0126 (20 μ M, 40 min) leads to a decrease in Ser²⁹⁵ phosphorylation (p<0.01).

D. The same treatment had no effect on PSD95 Thr¹⁹, phosphorylated *in vitro* by a different proline directed kinase or on the phosphorylation by TBK1 on the adjacent phosphorylation site, Ser⁴¹⁵ (Panel E).

F. Stimulation of PKA (forskolin, 50µM 10 min) of NR2B Ser¹³⁰³ phosphorylation (144%± 8, p<0.001) was not inhibited by CaMKII inactivation with KN-62 (10µM 30 min).

G. The same treatment (KN-62 10µM 30 min) reduces CaMKII autophosphorylation (p<0.001) and activity towards CaMKII substrate GluR1 pSer⁸³¹ (p<0.001).

Supplementary fig. 2. Phosphorylation of protein interaction domains and motifs.



A. Number of NMDA modulated peptides containing potential phosphorylation sites within different classes of short, functional motifs. Significantly enriched or depleted motifs denoted by + and – respectively (p, 0.05).

B. Number of phosphorylation sites located within different classes of short functional motifs. Significantly enriched or depleted motifs denoted by + and – respectively, +/- denoting a single class (e.g. SH2 ligand) comprised of multiple motifs, some enriched and others depleted.

Supplementary Table Legends:

Supplementary Table 1: Phosphopeptide identifications by LC/MS/MS and MS3

- A. MrExp (Experimental molecular mass)
- B. MrTh (Theoretical molecular mass)
- C. Charge (Peptide charge)
- D. Query (Spectrum query)
- E. Experiment
- F. Sequence (amino acid sequence)
- G. Var Mods (Variable modifications)
- H. Score (MASCOT ion score)
- I. Homology Threshold (MASCOT homology threshold score)
- J. Delta ppm (Delta between experimental and theoretical molecular mass, in parts per million)
- K. MS2-MS3 Pair? (Phosphopeptides with MS2 and MS3 spectral pairs)
- L. Spectrum (Fragment ion spectrum)

Note: Phosphopeptides approved with a FDR of < 1 % in the control (ExptX_C) and NMDA stimulated (ExptX_N) sample from experiments 1 and 2 are shown. MS2 and MS3 spectra are included and the MASCOT Ion score, Homology threshold and Delta ppm are shown for each phosphopeptide identification.

Supplementary Table 2: Summary of phosphorylation states identified in each phosphopeptide

- A. protein, gene name for proteins with phosphopeptides detected in MS
- B. peptide, amino acid sequences of detected phosphopeptides
- C. swprot, swissprot database identification number
- D. start, position in protein of first residue of peptide
- E. end, position in protein of last residue of peptide
- F. duplicated, duplicate samples
- G. single, single sample
- H. mixed, mixed samples
- I. NMDA effect, effect of NMDA stimulation on phosphorylated sequence
- J. composite peptide
- K. composite start,
- L. composite end,

Note: Also shown are phosphorylation states present and significantly enriched in NMDA/Control samples in both (F) and one (G) experiments, and those showing variable enrichment between experiments (H). For example, the entry in row 2 column F is 2 (ST) Control (0.36,-). This denotes that a doubly (ser/thr) phosphorylated state of the peptide was enriched in Control samples in both experiments, having NMDA/Control peptide intensity ratio 0.36 in one experiment, the peptide only present in Control samples in the other. Overall

effect of NMDA stimulation is given in column I (A = phosphorylated, B = dephosphorylated, C = unchanged, D = inconsistent/unclear). Overlapping peptides were grouped together if their shared amino acids contained all potential phosphorylation sites (pairs are highlighted in yellow).

Supplementary Table 3: List of NMDA-modulated proteins and their functional classification

Columns:

- A. protein, gene name for proteins modulated by NMDAR stimulation
- B. id, swissprot database identification number
- C. family, substrate functional classification
- D. subfamily, substrate functional classification
- E. N phosphorylated peptides, number of peptides phosphorylated per protein (column A), after NMDA treatment.
- F. N dephosphorylated peptides, number of dephosphorylated peptides per protein (column A), after NMDA treatment.
- G. NMDA effect, summary of the effect of NMDA stimulation.
- H. NRC/MASC, membership of NMDAR complex (NRC) or membrane associated guanylate kinase signaling complex (MASC) (0=absent, 1=present).
- I. ppid, protein protein interaction database identification number.

Supplementary Table 4: Analysis of glutamate receptors phosphorylation.

- A. protein, protein name
- B. phosphorylation site/sequence, amino acid residue of phosphoacceptor site or amino acid sequences of detected phosphopeptides
- C. NMDAR, effect of NMDAR stimulation
- D. mGluR(I), effect of mGluR type I stimulation. DHPG: 3,5-Dihydroxyphenylglycine
- E. D1, Effect of Dopamine type 1 receptor stimulation. 6-CIPB: (\pm)-6-Chloro-7,8-dihydroxy-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine
- F. PKA stimulation (FSK), effect of PKA stimulation by forskolin
- G. PKC stimulation (PdBu), effect of PKC activation by phorbol 12-13 dibutyrate

Note: Hippocampal slices were treated with 20 μ M NMDA (3 minutes), 100 μ M DHPG (15 minutes), 10 μ M 6-CIPB (15 minutes), 50 μ M forskolin (FSK) (15 min) or 10 μ M phorbol 12-13 dibutyrate (PdBu) (10min). See methods section. Unpaired t-tests were used to determine statistical significance ($p < 0.01$)

Supplementary Table 5: NMDA-modulated proteins linked to disease.

Columns:

- A. gene, gene name.
- B. disease, associated disease
- C. reference, Publication that reports gene/disease association.

Supplementary Table 6: Known (in vivo) phosphorylation sites used in peptide arrays

Columns:

- A. substrate, protein name.
- B. swprot, swissprot database identification number
- C. site, amino acid residue of phosphoacceptor site
- D. Peptide sequence, peptide used in array
- E. Peptide start, position in protein of first residue of peptide
- F. Peptide end, position in protein of last residue of peptide
- G. Pubmed PMID, Publication link that reports this site
- H. PMID site, the site referred to in the publication

Note: One peptide (for mKIAA0942) could not be aligned to the amino acid sequence in Supplementary Table 10. In this case, the peptide position is given relative to the site location in the supporting publication.

Supplementary Table 7: Phosphorylation of other sites in peptide array.

Columns:

- A. substrate, protein name.
- B. swissprot, database identification number
- C. site, amino acid residue of phosphoacceptor site
- D. N kinases, number of kinases phosphorylating each site
- E. kinases, name of kinases phosphorylating each site
- F. known site pmid, publication link that reports this site

Supplementary Table 8: Additional phosphorylation sites in synaptic proteins

Columns:

- A. substrate, protein name.
- B. swprot, swissprot database identification number
- C. site, amino acid residue of phosphoacceptor site
- D. Pubmed PMID, Publication link that reports this site

Note: phosphorylation sites (relative to sequence in Supplementary Table 10) and literature reference (PubMed id or link) for additional sites not utilized in peptide array.

Supplementary Table 9: Protein name and functional classification of substrates represented in peptide arrays assays

Columns:

- A. substrate, protein name.
- B. family, substrate functional classification
- C. subfamily, substrate functional classification
- D. swissprot id, database identification number
- E. swissprot species, database identification specie
- F. ensembl id, database identification number
- G. ensembl species, database identification specie
- H. human ensemble id, human database identification number

Supplementary Table 10: Protein sequences of substrates used in peptide arrays

Columns:

- A. substrate, protein name.
- B. swissprot id, database identification number
- C. sequence, substrates amino acid sequences

Supplementary Table 11: Protein kinases used in peptide arrays

Columns:

- A. kinase, kinase name.
- B. group, protein kinase classification
- C. family, protein kinase classification
- D. swissprot id, database identification number
- E. swissprot species, database identification species
- F. ensembl id, database identification number
- G. ensembl species, species

Supplementary Table 12: Peptide arrays quantitation

Columns:

- A. kinase, kinase used in peptide arrays
- B. substrate, substrate phosphorylated
- C. site, amino acid residue phosphorylated in peptide arrays
- D – F. sub-arrays (1,2,3), % incorporation shown for triplicate samples
- G. average (%), Mean for triplicate samples
- H. st. dev (%), standard deviation

Supplementary Table 13: Phosphorylation of known in vivo sites tested in peptide array.

Columns:

- A. substrate, protein name.

- B. swissprot, database identification number
- C. site, amino acid residue of phosphoacceptor site
- D. N kinases, number of kinases phosphorylating each site
- E. kinases, name of kinases phosphorylating each site

Note: there are 3 tables (Table 6, 7, 8) which report on kinase phosphorylation of peptides. Table 6 shows sites that are in vivo sites. Table 7 shows sites that are either not known to be in vivo sites (and are therefore novel or candidate in vivo sites) or known in vivo sites without the specific negative control for that particular site. Table 8 shows peptides that were phosphorylated without a specific negative control to identify the specific residue (more than one possible phosphoacceptor site).

Supplementary Table 14: Phosphorylation of sequences with non-determined phosphorylation sites.

Columns:

- A. substrate, protein name.
- B. swissprot, database identification number
- C. sequence, amino acid sequence with non determined phosphorylation site/s
- D. N kinases, number of kinases phosphorylating each sequence.
- E. kinases, name of kinases phosphorylating each sequence.

Supplementary Table 15: Phosphopeptide priming arrays:

Each table/box displays a title indicating the kinase used in the array.

Columns:

- A. protein, substrate name
- B. phosphorylation site, amino acid residue of phospho acceptor site
- C. peptide sequence, amino acid sequence containing the phospho acceptor site
- D. average (%), Mean for triplicate samples
- E. st. dev (%), standard deviation
- G. p, significance/p value

Note: the sites studied are highlighted in bold , previously modified (phosphorylated) sites are marked as: pS (phosphoserine) pT (phosphotreonine) and pY (phosphotyrosine). Average phosphorylation of at least 3 determinations is indicated and referred to the non modified sequence (100% phosphorylation).

Supplementary Table 16: List of sequences tested in phosphopeptide priming arrays:

Columns:

- A. substrate, protein name.
- B. site, amino acid residue of phosphoacceptor site
- C. sequence, amino acid sequences used in phosphopeptide priming arrays

Supplementary Table 17: Antibodies used in western blot assays

Columns:

- A. antibody, antibody target
- B. dilution, antibody working dilution used
- C. catalogue number
- D. company

In-house antibodies are not provided with company or catalogue number (X)

Supplementary Table 18: Recombinant kinases used in peptide arrays.

Columns:

- A. Recombinant kinase, kinase name/description
- B. Catalogue number, catalogue number (Upstate Biotechnology, Inc., Lake Placid, NY)

Supplementary Table 19: Kinase-kinase phosphorylation map of protein kinases used in peptide array assays.

Columns:

- A. kinase (substrate), name of protein kinase with phospho acceptor site
- B. site (human), amino acid residue of phosphoacceptor site in human
- C. site (mouse), amino acid residue of phosphoacceptor site in mouse
- D. function, effect of protein phosphorylation on kinase activity
- E. kinase (active), kinase responsible of substrate (column A) phosphorylation
- E. PMID, Publication link that reports this site

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