Online Supplemental Materials

Identification and characterization of neuronal MAP kinase substrates using a specific phosphomotif antibody

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1. Supplemental Discussion
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SUPPLEMENTAL DISCUSSION

Functional grouping of identified proteins
We used UniHI, an integrated online database of the human interactome (1), to map direct interactions between the proteins identified from cortical culture or rat brain PSD. In Supplemental Figure S5, the effect of OA-treatment on the amounts of affinity purified proteins is integrated into a color-coded protein-network that illustrates how MAP kinases could affect diverse functional systems. A large fraction of the putative MAPK substrates isolated by 5557 immunoprecipitation fell into several major functional groups.

Cytoskeleton
In the 5557 immunoprecipitates, we robustly identified microtubule-associated proteins MAP1B, MAP2 and tau (MAPT) (Supplemental Figure 4, shaded in green), which are known to be phosphorylated on multiples sites by proline-directed kinases ERK, JNK and CDK5 (Supplemental Table S1; see also Supplemental Table S2 for phosphopeptide sites). Additionally, we identified MAP1A and MAP6, which also have potential MAPK phosphorylation sites. JNK-mediated phosphorylation strengthens the binding of MAP1B and MAP2 to microtubules, and is required for proper dendrite arborization and neuritogenesis (2, 3). The microtubule associated serine/threonine kinases MAST1 and MAST2 were strongly enriched by OA in the 5557 immunoprecipitation, indicating increased phosphorylation. Both MAST1 and MAST2 contain PxS/TP motifs and we found a previously unknown proline-directed phosphorylation site in MAST1 (Supplemental Table S2). The roles of MAST1 and MAST2 in neurons are poorly understood.

Actin-associated proteins immunoprecipitated by 5557 phosphomotif antibody included: (i) the actin-binding proteins adducin 1 and 2 (ADD1 and ADD2) and ABLIM1, which might play a role in structural and functional synaptic plasticity (4); (ii) protein 4.1N, which may regulate GluR1 and dopamine receptor D2/3 trafficking (5, 6). The identification of specific proline-directed phosphorylation sites in adducin 1 offers a possible inroad into the regulation of actin cytoskeleton by MAPKs.
Supplemental Materials: Identification of neuronal MAPK substrates

**Ras/Rac1/MAPK signaling**

5557 immunoprecipitation of liprin-α3 (PPFIA3), G protein-coupled receptor kinase interactor 1 (GIT1), the Rac guanine nucleotide exchange factor βPIX (ARHGEF7) were all strongly increased (log₂(OA/untreated) from 1.9 to 2.5) by phosphatase inhibition. Interestingly, these proteins interact with each other and all contain multiple PxS/TP motifs, none of which have been reported to be phosphorylated so far. βPIX is a Rac1-GEF activated by the Ras/ERK/PAK pathway and regulates neurite outgrowth (7). GIT1 is a scaffold protein and ArfGAP that regulates ERK-signaling at focal adhesion sites (8) and is involved in spine morphogenesis (9, 10) and AMPA receptor trafficking together with liprin-α (11-13).

ERK phosphorylation of SOS1, a RasGEF found in PSD fractions, is reported to provide a negative feedback mechanism for Ras activation (14). A similar mechanism could apply for Abl interacting protein Abi1, which is crucial for Rac-dependent actin remodeling (15). Both SOS1 and Abi1 are strongly enriched upon phosphatase inhibition in 5557 immunoprecipitates.

**Cell adhesion**

The cadherin complex is important for presynaptic-postsynaptic adhesion (16), interacts with AMPA receptors and regulates synapse morphology and function (17-20). δ-catenin (CTNND2) and Plakophilin 4 (PKP4) were identified as putative MAPK substrates from both cortical culture and PSD preparations, and we sequenced multiple phosphorylation sites adjacent to proline in both proteins (Supplemental Table S2). These proteins belong to the p120 catenin family, which share 10 armadillo repeats interacting with the juxtamembrane region of cadherins. In neurons δ-catenin and PKP4 link cadherins to AMPA receptors via GRIP (21). δ-catenin is part of the chromosomal deletion region in cri du chat syndrome and δ-catenin knockout mice show severe cognitive impairment consistent with the human disorder (22, 23). It will be of interest to study how the cadherin complex is regulated by MAPK signaling.

**Scaffolds**

Scaffold proteins (Supplemental Figure 4, shaded in red) are the building blocks of the PSD and they regulate synaptic transmission (24, 25). The abundant scaffold PSD-
95/DLG4, with a defined phosphorylation site on serine-295, was identified as a probable MAPK substrate by 5557 immunoprecipitation from solubilized PSD (Table 2 and Supplemental Table S2). In the PSD, PSD-95 binds to GKAP/SAPAP family proteins, which in turn bind to Shank family scaffolds (25, 26). SAPAP2 and SAPAP4 (also known as DLGAP2/4) as well as Shank3 were identified in our proteomic analysis of MAPK substrates from the PSD (Table 2). MAGUIN/CNKS R2, another PSD scaffold, was strongly increased upon OA-treatment ($\log_2(OA/untreated) = 2.61$, Table I).

MAGUIN and its binding partner synaptic scaffolding molecule S-SCAM (also known as MAGI-2/AIP1), in which we identified a specific proline-directed phosphopeptide (Supplemental Table 2), were also identified from cortical cultures. S-SCAM/MAGI-2 also interacts with NMDA receptor subunits and multiple adhesion molecules like δ-catenin and neuroligin (27). Thus a network of key scaffold proteins in the PSD appears to be phosphorylated by MAPKs.

Neurabin1 (PPP1R9A) and spinophilin/Neurabin2 (PPP1R9B) are postsynaptic scaffolds for protein phosphatase 1 (PP1). OA treatment resulted in strong depletion of both proteins from 5557 immunoprecipitates ($\log_2$ scores were -2.3 and -1.9, respectively). To a lesser extent, OA also reduced 5557 immunoprecipitation of the catalytic subunits of PP1 (PPP1CA, PPP1CB, PPP1CC), which might indicate a general effect of OA on its target enzyme complex. PPP1R9A/Neurabin-1 has been shown to regulate spine morphology (28) and AMPA receptor dephosphorylation during LTD (29). It will be interesting to determine how MAPK phosphorylation affects neurabin activities.
SUPPLEMENTAL REFERENCES


Supplemental Materials: Identification of neuronal MAPK substrates

 synaptic transmission and long-term depression in hippocampus. *J Neurosci* 27, 4674-4686.


LEGENDS FOR SUPPLEMENTAL FIGURES AND TABLES

Supplemental Figure S1: Akt-phosphomotif and tyrosine phosphorylation in postsynaptic density fractions.
Immunoblot of rat brain fractions (5 µg, from Figure 2). Brain extracts were fractionated by discontinuous sucrose gradient and detergent extraction into post-nuclear supernatant (S1), cytosol plus light membranes (S2), crude synaptosomal fraction (P2) and sucrose gradient-purified synaptosomes extracted with 0.5% Triton X-100 (PSD1) and immunoblotted with antibodies detecting phospho-tyrosine and the Akt-phosphomotif (RxRxxS/T*).

Supplemental Figure S2: Concentration dependence of JNK inhibitor on 5557 phosphomotifs.
(A) Hippocampal neurons (DIV25) were incubated for 4 h with JNK inhibitor SP600125 at the indicated concentration. The extracts were immunoblotted with phosphoantibody 5557 and anti-PSD-95. (B) 5557 signal intensity was quantified by densitometry (bands above 85 kDa) and normalized to PSD-95 loading control (n = 12). Statistical analysis was performed with one-way ANOVA. ** denotes p < 0.01.

Supplemental Figure S3: Effect of kinase inhibitors on OA-induced phosphorylation in cultured neurons.
(A) Hippocampal neurons (DIV27) were pre-incubated for 10 min with kinase inhibitors as indicated (50 µM PD98059, 5 µM SB203580, 20 µM SP600125, 10 µM roscovitine) and then OA (0.1 µM) was added for 30 min. The extracts were immunoblotted with phosphoantibody 5557 and anti-PSD-95. PD+SB+SP denotes the combination of PD98059 (50 µM), SB203580 (5 µM) and SP600125 (20 µM). (B) 5557 signal intensity was quantified by densitometry (bands above 85 kDa) and normalized to PSD-95 loading control (n = 6). Statistical analysis was performed with one-way ANOVA. * denotes p < 0.05, ** denotes p < 0.01.
**Supplemental Figure S4: Representative LC-MS/MS runs.**
Global ion currents of a pair of gel pieces from 5557 immunoprecipitates of untreated and OA-treated cortical culture. The signal of the strongest ion current at every time point during peptide elution is shown. The global ion currents of the two comparative samples were highly similar indicating that the majority of proteins had not changed under the conditions.

**Supplemental Figure S5: Interactome of putative MAPK substrates.**
Interaction map of 5557 affinity purified proteins from cortical culture P2 with at least two peptides identified and rat brain PSD with at least one MAPK site predicted by Scansite (30, 31). Modified from UniHI (1). Proteins whose abundance increased or decreased by OA-treatment in purification from cortical culture are denoted by thick or dotted borders, respectively. Identification of a phosphopeptide containing S/T*P phosphorylation in this study (compare Supplemental Table S1) or in the PhosphoSite database (www.phosphosite.org) is denoted by bold font. Previously known MAPK substrates are enlarged. Proteins are grouped by function as indicated by the color code in the legend.

**Supplemental Table S1: Known MAPK and CDK5 substrates identified by MAPK motif phosphoantibody purification.**
Proteins identified in the cortical culture P2 and rat brain PSD sample (ut and OA refer to untreated and OA-treated cortical culture) that have been reported to be substrates of MAPK or CDK5, with references.

**Supplemental Table S2: Phosphopeptides identified by MS sequencing of proteins immunoprecipitated with 5557 phosphoantibody.**
Phosphopeptides identified by LC-MS/MS from untreated cortical culture (ut), okadaic acid treated culture (OA) or rat brain postsynaptic density (PSD). Due to partial trypsin cleavage, double phosphorylation or methionine oxidation (denoted by #) some phosphorylation sites were identified in distinct peptides multiple times. Amino acid residues are numbered according to the rat reference sequence (NCBI). The column “Known” denotes overlap with Trinidad et al. (32) (labeled T06) and Munton et al. (33)
(labeled M07). For each peptide, the MS/MS spectrum with the best XCorr score is available through a local link. N/A denotes peptides identified from the PSD-sample by re-analysis using LTQ for survey scans to improve sensitivity (see Experimental Procedures).

**Supplemental Table S3: Affinity purified putative MAPK-substrates identified and quantified by LC-MS/MS from cortical culture.**

5557 affinity purified proteins identified by LC-MS/MS from untreated and OA-treated cortical culture grouped into clusters based on shared peptides. Proteins are listed with the protein accession, GeneID and gene name (rat and human) from NCBI, the number of sequenced peptides, the sequence coverage from MS/MS data in percent and the enrichment factor upon OA-treatment ($\log_2$(OA/untreated) with standard error of mean). For convenience the full protein sequence downloaded from NCBI is included together with the number of predicted proline-directed phosphorylation sites for ERK, p38, CDK5 and cdc2 (Scansite, medium stringency). Putative phosphorylation sites and kinase binding motifs are summarized sorted by Scansite-score. For each protein the best (i.e. smallest) MAPK-prediction score is listed.

**Supplemental Table S4: Affinity purified putative MAPK-substrates identified and quantified by LC-MS/MS from rat brain PSD.**

5557 affinity purified proteins identified by LC-MS/MS from rat brain postsynaptic density grouped into clusters based on shared peptides. Proteins are listed with multiple NCBI identifiers, peptide number, sequence coverage from MS/MS data (in percent) and Scansite predictions for MAPK phosphorylation motifs as in Supplemental Table S3.

**Supplemental Table S5: Non-phosphorylated peptides identified by LC-MS/MS.**

For every identified peptide, only the spectrum with the best XCorr score was linked locally. N/A denotes peptides identified from the PSD-sample by re-analysis using LTQ for survey scans to improve sensitivity (see Experimental Procedures).
Supplemental Table S1: Known MAPK and CDK5 substrates identified by MAPK motif phosphoantibody purification.

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Supplementary Figure S1, Edbauer et al.
Supplementary Figure S2, Edbauer et al.
Supplementary Figure S3, Edbauer et al.
Retention Time (min)

Relative Abundance

Untreated sample

OA-treated sample

Supplementary Figure S4, Edbauer et al.
Figure S5