Abstract

Learning and memory processes are accompanied by rearrangements of synaptic protein networks. While various studies have demonstrated the regulation of individual synaptic proteins during these processes, much less is known about the complex regulation of synaptic proteomes. Recently, we reported that auditory discrimination learning in mice is associated with a relative down-regulation of proteins involved in the structural organization of synapses in various brain regions. Aiming at the identification of biological processes and signaling pathways involved in auditory memory formation, here, a label-free quantification approach was utilized to identify regulated synaptic junctional proteins and phosphoproteins in the auditory cortex, frontal cortex, hippocampus, and striatum of mice 24 h after the learning experiment. Twenty proteins, including postsynaptic scaffolds, actin-remodeling proteins, and RNA-binding proteins, were regulated in at least three brain regions pointing to common, cross-regional mechanisms. Most of the detected synaptic proteome changes were, however, restricted to individual brain regions. For example, several members of the Septin family of cytoskeletal proteins were up-regulated only in the hippocampus, while Septin-9 was down-regulated in the hippocampus, the frontal cortex, and the striatum. Meta analyses utilizing several databases were employed to identify underlying cellular functions and biological pathways. Data are available via ProteomeExchange with identifier PXD003089.

Keywords: auditory learning, chemical synapse, label-free quantification, learning and memory, phosphoproteomics, quantitative mass spectrometry.

The formation and maintenance of memories are generally assumed to be associated with changes of the synaptic proteome in relevant brain regions. Molecular mechanisms underlying these alterations include post-translational modifications, subcellular relocalization, and changes in the turnover of proteins (Lamprecht and LeDoux 2004; Bingol and Schuman 2005; Richter and Klann 2009; Rosenberg et al. 2014). Relocalization and shuttling of proteins between synaptic and non-synaptic sites as well as differential anchoring at cytoskeletal or cytomatrix structures have been reported as molecular correlates of synaptic activity or learning (e.g. Trinidad et al. 2013; Rao-Ruiz et al. 2015). Such rearrangement processes can be directly linked to activity-induced protein modifications like phosphorylation (Tweedie-Cullen et al. 2009). In a first systematic study on phosphoproteome dynamics, Li et al. (2014) reported changes in total phosphoproteome patterns after spatial memory formation in the hippocampus. Although not focused on synaptic protein fractions, this study revealed several synaptic phosphoproteins potentially regulated as long as 12 days after the initial learning.

Numerous studies have reported on the complexity of the synaptic proteome in general (Sheng and Hoogenraad 2007; Chua et al. 2010; Pielot et al. 2012; Wilhelm et al. 2014; Lassek et al. 2015). While several studies analyzed total proteome dynamics during processes of synaptic plasticity and learning (Henninger et al. 2007; Zheng et al. 2009; Monopoli et al. 2011; Meparishvili et al. 2015), to date, only few studies have addressed learning-induced dynamics of the synaptic proteome (Jüch et al. 2009; Kähne et al. 2012; Rao-Ruiz et al. 2015).

Formation of a memory trace occurs in multiple stages with differential dependencies on protein synthesis and/or modifications and involves a complex interplay of various brain regions (for review, see Matthies 1989; McGaugh 2000; Kandel et al. 2014). In mammals, cognitively demanding learning processes engage subregions of the cerebral cortex connected with subcortical areas. Auditory discrimination learning, for example, involves, among others, projections from the auditory cortex (AC) to the striatum (STR), the main reinforcement-analyzing structure (Schultz 2010), and leads to potentiation of corticostral synapses (Xiong et al. 2015) ultimately entailing a task-dependent increase in corticostral communication (Schulz et al. 2016). Furthermore, auditory learning is accompanied by increased spine formation within the AC with newly formed spines persisting at least for several days, thus, potentially forming a structural correlate for the memory trace (Moczulska et al. 2013). A well-characterized example of auditory cortex-dependent learning is the frequency-modulated tone discrimination (FMTD) task, a go/no-go shuttle box avoidance paradigm that involves the AC as crucial interpreter of task-specific features of auditory information (Ohl et al. 1999, 2001; Rybalko et al. 2006; Scheich et al. 2011). In the AC and in the frontal cortex (FC), which is reciprocally connected with the AC, acquisition of the FMTD task elicits a rise in dopamine levels pointing to a role of cortical dopamine in synaptic remodeling (Stark and Scheich 1997; Stark et al. 2004; Ilango et al. 2012; Oli 2015). Retention of the newly acquired FM discrimination is critically controlled by dopamine-mediated protein synthesis in the AC (Schicknick et al. 2008; Reichenbach et al. 2015).

Previous studies with the inhibitors anisomycin and rapamycin injected into the AC identified two distinct protein synthesis-dependent mechanisms required immediately after encoding for the initial maintenance and long-term consolidation of FMTD memories (Kraus et al. 2002; Tischmeyer et al. 2003). In this study, we were interested in analyzing subsequent changes (24 h after training) in the synaptic proteomes and phosphoproteomes of brain regions involved in FMTD learning, i.e. the AC, FC, and STR. We also included the hippocampus (HIP) as central memory hub and key structure in the acquisition of active avoidance learning in the shuttle box (Myhrer 1976; Pavlova et al. 2010), which is potentially implicated in FMTD learning (Ilango et al. 2014). The AC is indirectly connected to the HIP via the entorhinal cortex (important functional connections of the rodent AC are sketched in Fig. 1; for details, see Budinger and Scheich 2009).

Building on our previous study (Kähne et al. 2012), which showed that proteomic changes after differential conditioning of mice to FM s occur in the above mentioned four brain regions, we addressed here the following questions: (i) Are proteomic changes of global nature or rather brain region-specific? (ii) Can changes in phosphorylation patterns be identified 24 h after training? (iii) What are the potential cellular processes and biological pathways represented by these changes? To this end, we performed quantitative analyses of changes in the composition of synaptic junction (SJ) preparations in mice 24 h after auditory discrimination conditioning. The study revealed distinct rather than common patterns of proteomic and phosphoproteomic synaptic changes in the analyzed brain regions. Moreover, it confirms earlier findings that among all regulated SJ proteins more are down- than up-regulated in their relative abundance (Kähne et al. 2012).

Materials and methods

Animals and behavioral experiments

Male 10–16-week-old C57Bl/6J mice were used for this study. They were housed and treated as described (Kähne et al. 2012). Animal experiments were performed in accordance with the regulations of the German Federal Law, the respective EU regulations, and NIH guidelines, and were approved by the Landesverwaltungsamt Hallesalz for the State Saxony-Anhalt.

FMTD training: mice were trained in a two-way shuttle box go/ no-go task to discriminate between sequences of rising (4–8 kHz,
CS+) and falling FM tones (8–4 kHz, CS−) to avoid mild foot-shock (AV group) as previously described (Kähne et al. 1999). The AC is connected with a number of cortical and subcortical structures (Budinger and Scheich 2009), including strong efferent projections to various subcortical structures. The connection between AC and the frontal cortex (FC) is reciprocal. Strengths of connections are roughly indicated by the thickness of arrows.

For preparation of protein samples enriched for synaptic junctional structures (SJ-enriched samples, Smalla et al. 2000, 2012; Bonn et al. 2007), tissue was homogenized in 300 μL of 10 mM Tris/HCl, pH 8.1, 0.5% Triton X-100 containing protease and phosphatase inhibitors. After incubation for 1 h at 4°C, samples were centrifuged at 100 000 g for 1 h. The resulting pellets were rehomogenized in the same buffer and centrifuged again. The final pellets were resuspended in 500 μL of deionized water and stored at −80°C. Aliquots were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) to adjust protein amounts for proteome analysis. Samples of set 1a were processed as described below for proteome analysis; samples of set 2a were used for immunoblotting and/or trypsin-digested for MRM (Supporting Information).

The adjusted SJ-enriched samples of set 1a were heated in SDS-sample buffer, and 4 μl aliquots were separated by SDS-PAGE (3% stacking gel, 12% separation gel) in a Mini PROTEAN System (Bio-Rad Laboratories, Hercules, CA, USA). After Coomassie staining, gel pieces covering the molecular weight range of ~35–170 kDa were manually excised. In-gel digestion was performed according to Shevchenko et al. (1996) with slight modifications: gel pieces were washed twice in 0.1 M NH₄HCO₃ followed by 100% acetonitrile (ACN). Afterwards, cysteine residues were carbamidomethylated by dithiothreitol and subsequent iodoacetamide treatment. Gel pieces were washed twice again, dried, rehydrated in freshly prepared digestion buffer, i.e. 50 mM NH₄HCO₃ containing 12.5 ng/μl of trypsin (Promega, Madison, WI, USA, Trypsin Gold), and incubated at 37°C overnight. Tryptic peptides were extracted with 25 mM NH₄HCO₃ followed by ACN under sonication and lyophilized.

Phosphopeptide-enriched and phosphopeptide-depleted fractions were prepared from the tryptic peptides of the SJ-enriched samples by TiO₂ chromatography (adapted from Thingholm et al. 2008). Briefly, tryptic peptides were dissolved in 80% ACN/2.5% trifluoroacetic acid (TFA) and mixed with ~2 mg Tisphane TiO₂-beads (GL Sciences, Tokyo, Japan). After incubation for 1 h at ~20°C, the beads were spun down (16 000 g, 1 min) and the supernatants were collected. The beads were washed in 80% ACN/2.5% TFA, followed by 80% ACN/0.1% TFA and 0.1% TFA, and the combined supernatants were lyophilized. They represented the phosphopeptide-depleted fraction and were redissolved prior to proteome analysis. The TiO₂-beads were finally washed with 400 mM NH₄OH/30% ACN and the resulting eluates were concentrated in a vacuum centrifuge to a volume of ~6 μl for proteome analysis (phosphopeptide-enriched fraction).

**Proteome analysis**

Phosphopeptide-depleted and phosphopeptide-enriched fractions from SJ-enriched protein samples were analyzed by high-resolution mass spectrometry (MS) to allow label-free quantification and reliable discovery of post-translational modifications. LC-MS/MS was performed on a hybrid dual-pressure linear ion trap/orbitrap mass spectrometer (LTQ Orbitrap Velos Pro, Thermo Scientific, San Jose, CA, USA) equipped with an EASY-nLC Ultra HPLC (Thermo Scientific). For analysis, peptide samples were adjusted to 10 μl 2% ACN/0.1% TFA and fractionated on a 75 μm (inner diameter), 25 cm PepMap C18-column, packed with 2 μm resin (Dionex,
Thermo Scientific). Separation was achieved through applying a gradient from 2 to 35% ACN in 0.1% formic acid over 150 min at a flow rate of 300 nL/min.

An Orbitrap full MS scan was followed by up to 10 LTQ MS/MS runs using collision-induced dissociation (CID) fragmentation of the most abundantly detected peptide ions. Essential MS settings were as follows: full MS (FTMS; resolution 60 000; m/z range 400–2000); MS/MS (Linear Trap; minimum signal threshold 500; isolation width 2 Da; dynamic exclusion time setting 30 s; singly charged ions were excluded from selection). Normalized collision energy was set to 35%, and activation time to 10 ms. Minimum information about the proteomics experiments (MIAPE-MS and MSI) is provided in Tables S1a and S1b. MS proteomics data have been deposited to the ProteomeXchange Consortium (Vizcaino et al. 2014) via the PRIDE partner repository with the dataset identifier PXD003089 and 10.6019/PXD003089.

MRM and/or immunoblotting were employed to paradigmatically verify the training-induced regulation of SJ-enriched proteins selected from Table S3 using mice of set 2a (see above). Details and results are given in Tables S1c, S1d, and S5 (for MRM), and in Figure S3 and Tables S2 and S5 (for immunoblotting).

Data analysis
Raw data processing, protein identification, and phosphopeptide assignment of the high-resolution Orbitrap data sets were performed using ProteomeDiscoverer 1.4 (Thermo Scientific). For a combined database search, Sequest and Mascot algorithms were used. False discovery rate was calculated by Percolator 2.0.4 algorithm and set to <1%. Label-free quantification was performed using PEAKS Studio V.7.0 label-free algorithm (Bioinformatics Solutions, Waterloo, Canada). The resulting data were re-evaluated using Progenesis LC-MS software package (Nonlinear Dynamics/ Waters, Newcastle, UK). Phospho-site determination was performed by phosphoRS 3.0 (Taus et al. 2011) and the resulting data were evaluated using the Progenesis LC-MS software package. Proteins with abundance ratios (AV/NV) of >1.5 or <1/1.5 at p < 0.05 were considered as significantly regulated. Note: because of losses during SJ preparation and/or nanoLC-MS/MS, the numbers of individual animal data sets were diminished for AC to n = 4 per group and for FC to n = 5 in the AV group. MRM data were analyzed using Skyline (MacLean et al. 2010).

Functional annotation and network analysis of functional interactions of differentially regulated proteins were done using the web-based tool GeneCodis (Tabas-Madrid et al. 2012) and QIAGEN’s Ingenuity Pathway Analysis (IPA, QIAGEN Redwood City, www.qiagen.com/ingenuity). Single enrichment analysis by GeneCodis utilized functional annotations of the Gene Ontology (GO) database, Kyoto Encyclopedia of Genes and Genomes, Protein ANalysis THrough Evolutionary Relationships (PANTHER) and InterPro (EMBL-EBI, Hinxton, UK). As control, the whole mouse proteome was used, the cut-off was at p < 0.05 (hypergeometric distribution) and p-values were corrected by the false discovery rate method of Benjamini and Hochberg (1995). Detection of biological pathways was performed by IPA, which uses the Ingenuity Knowledge database for annotation. Significance was calculated by Fisher’s exact test and the multiple testing corrected p-values were obtained by the Benjamini–Hochberg method.

Results
We analyzed changes in the synaptic proteomes of four brain regions implicated in FMTD learning (i.e. AC, FC, HIP, STR; Fig. 1). Our previous study on the same learning paradigm utilizing high-throughput low-resolution MS of a gel-free separated synaptic proteome revealed primarily high molecular weight cytoskeleton-associated proteins as regulated (Kähne et al. 2012). This study has been based on SDS-gel separation of SJ-enriched protein fractions, high-resolution Orbitrap MS, and label-free quantification. This strategy allows the confident detection and quantification of medium molecular weight proteins and their comparative profiling in mice 24 h after FMTD training to avoid mild foot-shock (AV group) and naive control mice (NV group). For technical reasons, proteins smaller than 35 kD were not resolved by our gel system and accordingly not identified. The excluded protein fraction may also contain learning-relevant proteins that will require separate attention. Both phosphopeptide-depleted and phosphopeptide-enriched fractions of SJ protein samples were analyzed. For protein quantification, data of both fractions were combined. Detailed information on proteins with significant changes in protein abundance and phosphopeptide occurrence in SJ-enriched fractions is summarized in Tables S3 and S4, respectively.

Common and brain area-specific dynamics of SJ-enriched proteomes
Reliably quantifiable information was acquired for a total of 67 306 peptides, representing 6272 proteins from all four brain regions analyzed (Fig. 2). Altogether, 3033 different proteins were identified within the four SJ-enriched preparations. About 55% and 50% of these proteins are listed in the SynProt database of synaptic proteins (www.synprot.de; Pielot et al. 2012) and the ‘Genes-to-cognition’ database (https://www.genes2cognition.org/), respectively. Volcano plots demonstrating the relative abundance profiles of all proteins deduced from quantifiable peptides revealed significant training-induced proteome differences in each of the analyzed brain regions (Fig. 2). In total, the relative abundance of 7369 of the quantified peptides showed significant differences in the SJ-enriched samples from AV mice when compared to the corresponding samples from NV controls. This corresponds to 4791 protein changes within all analyzed brain regions. Considering that some of these proteins were regulated in multiple regions (Table S3), the changes concern 359 different proteins. About 79% of these regulated proteins are listed in SynProt and 72% in ‘Genes to Cognition’, documenting the enrichment of synaptic and/or cognition-relevant proteins in the analyzed samples.

1459 after removal of artifacts and double database entries.
At the level of protein abundance, 7.6% of all quantified proteins showed significant training-induced changes. To further examine this observation, cluster analyses of these significantly regulated proteins were performed (heat maps in Fig. 2). As detailed in Table S3, statistical analysis revealed eight up- and 51 down-regulated proteins in the AC, four up- and 126 down-regulated proteins in the FC, 76 up- and 86 down-regulated proteins in the HIP, and 35 up- and 73 down-regulated proteins in the STR. Thus, with the exception of the HIP, which showed a rather balanced regulation profile, i.e. comparable numbers of up- and down-regulated proteins, a preference for down-regulation among the regulated proteins was observed in the other brain regions.

To assess cross-regional and brain region-specific profiles of training-induced synaptic proteome changes, a Venn analysis was performed (Fig. 3). It turned out that 78 (22%)
of the 359 significantly regulated proteins showed changes across two or more of the analyzed brain regions. For two proteins, CYFIP2 (Cytoplasmic FMR1-interacting protein 2, CYFP2_MOUSE; Abekhoukh and Bardoni 2014), and the myelin proteolipid protein (PLP, MYPR_MOUSE), significant abundance changes were detectable in all four brain areas. The general down-regulation of PLP, a protein mainly produced by oligodendrocytes, is surprising. However, PLP is routinely found associated with synaptic protein fractions including the postsynaptic core complex (www.synprot.de; Fernandez et al. 2009) and its regulation was confirmed by MRM analysis (see Table S5).

Eighteen proteins were regulated in the SJ-enriched fractions of three of the investigated brain regions; 15 of them, including the actin modulator PHAR1/Phactr1 (Phosphatase and actin regulator 1, PHAR1_MOUSE; Allen et al. 2004), the guanosine triphosphate (GTP)-binding cytoskeletal component Septin-9 (SEPT9_MOUSE; Mostowy and Cossart 2012), the cell adhesion molecule IgSF8 (Immunoglobulin superfamily member 8, G3UYZ1_MOUSE; Ray and Treloar 2012) as well as a number of RNA-binding and -modifying proteins (Table S3), showed regionally concordant changes. Altogether, 58 proteins are regulated in two of the four regions analyzed (Fig. 3), of which 54 proteins showed regionally concordant changes.

Changes of the vast majority (78%) of significantly regulated proteins after FMTD training were restricted to individual brain regions (Fig. 3) implying a rather autonomous brain area-specific regulation of SJ proteins. This notion is supported by the finding that several of the identified proteins are differentially regulated in different brain regions (Table S3). These include CYFIP2 (down-regulated in AC, FC, and STR, up-regulated in HIP), the scaffolding protein of the postsynaptic density (PSD) Shank3/ProSAP2 (SH3 and multiple ankyrin repeat domains protein 3, SHAN3_MOUSE; down in FC, up in HIP and STR), and AKAP5 (A-kinase anchor protein 5, AKAP5_MOUSE; down in AC and FC, up in STR) (Sheng and Hoogenraad 2007), as well as the membrane fission promoting GTPase Dynamin-1 (DYN1_MOUSE; up in AC, down in FC and STR) (Ferguson and De Camilli 2012). PHAR1/Phactr1 is down-regulated in FC, HIP, and STR, but to different degrees (Figure S2a).

On the other hand, we find paralogous proteins regulated in a similar way in the same brain region. For instance, members 1 and 3 of the Homer family of postsynaptic scaffolding and adaptor proteins (HOME1_MOUSE, HOME3_MOUSE; Sheng and Hoogenraad 2007) are up-regulated in HIP (Fig. S2b). Similarly, members of the Septin family of GTP-binding cytoskeletal-like proteins (Mostowy and Cossart 2012) are primarily regulated in HIP. Here, the isofoms 2, 5, 6, 8, 11 are up-regulated. In contrast, Septin-9 is down-regulated in HIP as well as in FC and STR (Fig. S2b).

Glial proteins have also been detected in the SJ-enriched samples as differentially regulated after auditory discrimination training. This includes regulation of the astroglial proteins ezrin (EZRI_MOUSE, down), glutamine synthetase (GLNA_MOUSE, up), and aquaporin-4 (Q8BR89_MOUSE, down) specifically in the HIP (Table S3). In contrast, the aforementioned PLP (MYPR_MOUSE) was down-regulated in all four brain regions.

**Region-specific regulation of phosphopeptides in SJ-enriched protein fractions**

Memory processes are associated with a variety of protein modifications, including changes in protein phosphorylation (e.g. Giese and Mizuno 2013; Rosenberg et al. 2014). We

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**Fig. 3** Cross-regional and region-specific proteome and phosphoproteome changes. The numbers of significantly regulated SJ-enriched proteins of the analyzed brain regions after FMTD training are compared in a Venn diagram. Numbers refer to proteins with training-induced abundance changes in SJ-enriched protein fractions and numbers in parentheses to proteins with changed phosphorylation patterns upon FMTD training. Venn analysis was performed with the mathematical software package ‘DanteR’ (Pacific Northwest National Laboratory/http://omics.pnl.gov) using data from Tables S3 and S4. AC, auditory cortex; FC, frontal cortex; HIP, hippocampus; STR, striatum.
used TiO₂-based enrichment of phosphopeptides to study changes in the synaptic phosphoproteome after FMTD training. In total, 4208 phosphopeptides have been identified, originating from 1905 different proteins. The abundances of 58 phosphopeptides in the SJ-enriched protein fraction exhibited significant differences between trained and naïve mice. Most of these modifications (88%) were detected in individual brain regions only (Fig. 3). A detailed list of significantly regulated phosphopeptides and the deduced proteins is given in Table S4. Interestingly, 53 out of the 58 proteins with training-related phosphopeptide changes did not exhibit significant simultaneous changes of their protein abundance in the SJ-enriched protein fraction of the same brain region. Moreover, four proteins in the FC, i.e. two proteins associated with membrane transport processes (AP2-associated protein kinase 1, AAK1_MOUSE; Conner and Schmid 2002; AP3 complex subunit beta-2, AP3B2_MOUSE, Faundez et al. 1998), an RNA-binding protein (Matrin-3, MATR3_MOUSE; Salton et al. 2011), and the actin-associated protein β2-spectrin (Spectrin beta chain, non-erythrocytic 1, SPTB2_MOUSE), showed opposite changes at the two detection levels. This indicates that training-induced changes in phosphopeptide abundance do not simply reflect changes in the abundance of a given protein in the SJ-enriched fractions but also, at least in part, changes in mechanisms of posttranslational modification (phosphorylation/dephosphorylation) of such proteins. Protein modifications in turn can regulate the association with the SJ protein networks (Sheng and Hoogenraad 2007; Sheng and Kim 2011). Strongest up-regulation of phosphopeptide abundances was observed in β2-spectrin, the PSD adaptor protein DLGAP3/SAPAP3 (Disks large-associated protein 3, DLGP3_MOUSE, Welch et al. 2007), the BAR-domain protein amphiphysin (AMPH_MOUSE) involved in endocytosis (Ren et al. 2006), and the non-receptor tyrosine kinase Src (neuronal proto-oncogene tyrosine-protein kinase Src, SRC_MOUSE, Kalia et al. 2004) in FC. Strong reduction of phosphorylation after training was detected in HIP for the melanoma-associated antigen E1 (MAGE1_MOUSE) and for transmembrane protein 64 (TMM64_MOUSE), a protein potentially involved in Ca²⁺ signaling (Kim et al. 2013), and in FC for TrkC (NT-3 growth factor receptor, NTRK3_MOUSE) (Table S4).

**Functional annotation of regulated proteins**

To gain insight into cell biological functions underlying the observed proteome changes, we assigned functional categories to the regulated proteins on the basis of the SynProt database (www.synprot.de). In Fig. 4, the numbers of up-versus down-regulated SJ-associated proteins and phosphoproteins are documented; detailed information is specified in Tables S3 and S4 with respect to detection levels, brain regions, and functional categories. At the level of protein abundance in SJ-enriched fractions (Fig. 4a), members of the categories ‘cytoskeleton, scaffolding, extracellular matrix’ (ECM), ‘cell adhesion’ and ‘DNA and RNA binding, transcription, translation’ were among the most frequently regulated proteins. Components of other functional categories, including those with putative functions in inter- and intracellular communication, such as ‘endocytosis, exocytosis, trafficking’ and ‘signal transduction’, were recognized as well, but to a lesser extent. In contrast, at the level of phosphopeptide abundance (Fig. 4b), the most prominent effects of auditory discrimination training were evident in the category ‘signal transduction’. The lists of proteins showing training-induced regulation of phosphopeptide abundance (Table S4) includes neurotransmitter and neurotrophin receptors as well as regulators and scaffolds of the synaptic cytomatrix (DLG2, DLGAP3, β2-spectrin, AP-3 complex subunit β2, AP-2-associated protein kinase 1).

To assess biological processes mostly affected by FMTD training, we utilized GeneCodis (Tabas-Madrid et al. 2012), which performs a single enrichment analysis based on different annotation databases. Data on abundances of proteins and phosphopeptides (Tables S3 and S4) were combined for the analysis, but brain regions were considered separately. To avoid redundant annotations, we took only those terms from the GO database into account that describe biological processes. A graphic overview depicting clustering of GO terms is displayed in Fig. 5 based on data summarized in Table S6. Strikingly, different cellular transport processes including ion transport across membranes as well as intracellular protein and membrane transport (e.g. endo- and exocytosis), are significantly regulated. In addition, alterations in the SJ-enriched proteome indicate that RNA-based processes, including local protein biosynthesis as well as diverse metabolic and signaling pathways, seem to be involved in learning-dependent restructuring of synapses. Most interestingly, processes of cellular development, learning, locomotory exploration behavior, and synaptic transmission were also signified by the observed proteomic pattern changes.

Further, IPA was used to identify canonical pathways represented by the proteomic changes 24 h after the learning experiment. Table 1 lists 11 pathways with highest significance levels. While more general pathways like ‘clathrin-mediated endocytosis signaling’ and ‘remodeling of adherens junctions’ were altered in all the brain regions investigated, more specific canonical pathways were identified in individual areas. ‘GABA receptor signaling’ representing components of the main inhibitory system was significantly affected in the cortical subfields AC and FC as well as in the STR, but not in the HIP. On the other hand, ‘glutamate receptor signaling’ and the related pathway of ‘synaptic long-term potentiation (LTP)’ were identified as key pathways in the HIP, a prime target region for learning-related LTP. Intriguingly, in the STR, a central area of the dopaminergic reinforcement-analyzing system, the ‘dopamine receptor
signaling’ pathway turned out to be significantly influenced. Finally, ‘Notch signaling’ and ‘calcium signaling’ were identified with significance in the FC only. It should be noted that even those cellular processes and pathways found to be regulated in all analyzed brain regions were represented by area-specific changes of individual components (e.g. Figure S4) pointing to additional regional specificity of the induced biological processes and their underlying signaling networks.

Discussion
The findings of this study suggest that training mice to discriminate the modulation direction of FM tones in order to avoid mild foot-shock reduces the association of proteins with synaptic cytomatrices in the cortical, hippocampal, and striatal brain samples 24 h later. At the level of protein abundance, less than 8% of 6272 quantified proteins showed significant training-induced changes. Most of these alterations were detected in individual brain regions; only 20 proteins show significant regulation in three or four of the brain areas investigated. At the phosphopeptide level, ≈ 3% of 1905 identified proteins were significantly regulated 24 h after FMTD training. Again, the vast majority of these modifications were restricted to individual brain regions. Most of the proteins found to be regulated in particular regions were identified in other regions as not significantly regulated, suggesting brain region-specific effects of FMTD training.

Protein networks underlying FMTD learning
The actin cytoskeleton and its regulatory proteins participate in learning-dependent synaptic plasticity processes subserving various types of memories (for review, see Lamprecht 2014). Consistent with previous findings regarding the molecular signature of the FMTD learning paradigm (Kähne et al. 2012), this study, focusing on a different set of synaptic proteins, emphasizes the involvement of proteins crucial for the re-organization of cellular structure. Relative down-regulation of the majority of regulated SJ-enriched proteins suggests that learning processes may initially loosen the association of proteins with synaptic cytomatrices to facilitate subsequent long-lasting plastic rearrangements (for discussion, see Kähne et al. 2012). A network of FMTD training-regulated proteins based on the IPA networks ‘cellular assembly and organization’ and ‘cell morphology’

Fig. 4 Assignment of FMTD training-regulated SJ-enriched proteins to structural and functional elements and processes. (a) Numbers of proteins regulated in their abundance as listed in Table S3. (b) Numbers of proteins with phosphopeptide changes as listed in Table S4. AC, auditory cortex; FC, frontal cortex; HIP, hippocampus; STR, striatum.
revealed a number of actin cytoskeletal elements and interacting components of the PSD (Figure S4) – many of them known to be involved in synaptic plasticity and assembly. Among these, Shank3/ProSAP2, AKAP5, and PHAR1/Phactr1 are found regulated in three of the four investigated brain areas. Shank3 is known as major postsynaptic scaffold protein interacting directly or indirectly with a variety of membrane and cytoplasmic proteins (Gundelfinger et al. 2006). AKAP5 is a scaffold for a major signaling complex in excitatory synapses (Sanderson and Dell’Acqua...
Fig. 5 Representation of biological functions and pathways in the identified protein data sets by single enrichment analysis (SEA) utilizing GeneCodis as a tool (see Table S6). The analysis was performed for each examined brain region with the combined data on protein and phosphopeptide abundances (Tables S3 and S4). Annotation databases such as Gene Ontology (GO) (http://geneontology.org/) assign proteins and genes to their ‘biological functions’ or ‘biological pathways’. The SEA tests for significantly represented biological pathways within a given list of proteins. The significance is determined by comparing the frequency of representation of a given pathway in that list with the expected random frequency. The network was calculated by in-house scripts and visualized with the tool Gephi (http://gephi.github.io/). Only GO terms with at least three proteins in at least one of the brain regions were taken into account. Nodes are depicted as circles. Each node represents a GO term, the color indicates the brain region (green: auditory cortex; blue: frontal cortex; magenta: hippocampus; red: striatum). The size of a given node visualizes the number of proteins that it has in common with other nodes. Additionally, the number and strengths of connections (indicated by line width) indicate the number of proteins that a particular node shares with other nodes. Numbers and strengths of connections, in turn, determine the distance of nodes, thus clustering closely related nodes. If nodes represent the same GO term and include identical sets of proteins, but derive from different brain regions, they were merged into one node (indicated by respective colors).

Table 1 Top canonical pathways represented by identified changes in the synaptic proteomes in individual brain regions

<table>
<thead>
<tr>
<th>Top canonical pathways</th>
<th>Auditory cortex</th>
<th>Frontal cortex</th>
<th>Hippocampus</th>
<th>Striatum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p-value</td>
<td>n/n_{tot}</td>
<td>p-value</td>
<td>n/n_{tot}</td>
</tr>
<tr>
<td>Clathrin-mediated endocytosis signaling</td>
<td>3.31E-03</td>
<td>4/185</td>
<td>9.11E-08</td>
<td>11/185</td>
</tr>
<tr>
<td>Axonal guidance signaling</td>
<td>0.422</td>
<td>2/433</td>
<td>3.57E-02</td>
<td>7/433</td>
</tr>
<tr>
<td>Calcium signaling</td>
<td>0.118</td>
<td>2/178</td>
<td>3.87E-02</td>
<td>4/178</td>
</tr>
<tr>
<td>Regulation of cellular mechanics by calpain protease</td>
<td>– –</td>
<td>– –</td>
<td>0.335</td>
<td>1/57</td>
</tr>
<tr>
<td>RhoA signaling</td>
<td>0.333</td>
<td>1/122</td>
<td>0.216</td>
<td>2/122</td>
</tr>
<tr>
<td>Notch signaling</td>
<td>– –</td>
<td>– –</td>
<td>2.49E-03</td>
<td>3/38</td>
</tr>
<tr>
<td>Remodeling of epithelial adherens junctions</td>
<td>2.14E-02</td>
<td>2/68</td>
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<td>Dopamine receptor signaling</td>
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<td>– –</td>
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<tr>
<td>Synaptic long-term potentiation</td>
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<td>0.208</td>
<td>2/119</td>
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For this analysis QIAGEN’s Ingenuity® Pathway Analysis (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity) was used: Selection criteria were minimum significance levels < 0.05 and at least three proteins per pathway in at least one brain region. n/n_{tot} – The table gives the number of regulated proteins identified in the proteomics screen (n) in relation to the total number of proteins in the reference proteome of the defined Ingenuity® pathway (n_{tot}). p-values indicating significant overlaps are given in bold. For the analysis, the combined data of synaptic protein and phosphopeptide abundances (Tables S3 and S4) were used.


2011) and Phactr1 is a G-actin and phosphatase-1-binding protein (Allen et al. 2004). Another very interesting protein regulated in all four analyzed brain areas is the cytoplasmic fragile X mental retardation protein (FMRP)-interacting RNA-binding protein CYFIP2. CYFIPs are also part of the WAVE complex and thus involved in Rac signaling to trigger Arp2/3-dependent actin nucleation. This process is known to be important in the spatiotemporal regulation of actin dynamics in cell adhesion and vesicle trafficking as well as synaptic development and plasticity (Abekhouch and Bardoni 2014). Shank3 indirectly acts on the WAVE complex (Figure S4; Han et al. 2013). Phactr1 is supposed to play also an important role in actin reorganization (Allen et al. 2004; Allain et al. 2012) and to make contact with the central PSD scaffolding protein PSD-95 (DLG4) (Arbuckle et al. 2010). In addition to the widely regulated CYFIP2, Shank3, and Phactr1, some elements of the actin-associated machinery were modulated differentially in individual brain areas (Figure S4b–e). For example, in the FC, the actin-associated proteins β-Adducin (Q8C0Y2-MOUSE), Wiskott–Aldrich syndrome family member 1 (WASF1/WAVE1, WASF1_MOUSE), Cortactin-binding protein 2 (CTTBP2, CTTB_MOUSE), and Cortactin-binding protein N-terminal-like protein (CTTBP2NL, CT2NL_MOUSE) were specifically regulated (Figure S4c). All four proteins have been implicated in dendrite and spine plasticity and in learning and memory processes (Soderling et al. 2003, 2007; Rabenstein et al. 2005; Bednarek and Caroni 2011; Ruediger et al. 2011; Chen and Hsueh 2012; Chen et al. 2012; Shih et al. 2014). Moreover, neurotransmitter receptors, including GluA2- and GluN2B-containing glutamate receptors and the D1 dopamine receptor are synaptic membrane proteins linked to the subsynaptic protein network and differentially regulated in the brain structures analyzed (Figure S4b–e).

Septins constitute a family of proteins regulated 24 h after the learning experiment. These proteins are considered as the ‘fourth component of the cytoskeleton’ (Mostowy and Cossart 2012) involved in the coordination of actin and microtubule remodeling in neurites, lateral compartmentalization of membranes, and entry of membrane proteins into...
dendritic spines (Caudron and Barral 2009; Hu et al. 2012; Ageta-Ishihara et al. 2013; Ewers et al. 2014). In the CNS, septins are abundant and exhibit cell type-specific and developmentally regulated patterns of expression (Finger et al. 2003; Shinoda et al. 2010). Interestingly, septin-5, 6, and 11, which were up-regulated in the hippocampus, are localized at dendritic branch points and at the base of dendritic spines (Xie et al. 2007). Depletion of septin-6 or -11 reduces dendritic arborization and alters the length, density, and head morphology of dendritic spines (Cho et al. 2011).

Considering the intimate physical and physiological connection of astrocytes and neurons at synaptic junctions, it is not surprising that also specific astroglial proteins have been detected and found to be differentially regulated after FMTD training. Of particular interest might be the differential regulation of the astroglial proteins ezrin (down), glutamine synthetase (up), and aquaporin-4 (down) specifically in the HIP. The actin cytoskeleton-associated protein ezrin is a key component of peripheral astrocytic processes (PAP) associated with synapses and critical for motility of these fine processes and thereby the structural plasticity of PAPs (Lavialle et al. 2011). In conjunction with the down-regulation of aquaporin-4, which is generally positively correlated with astroglial volume and may regulate adaptive swelling’ of PAPs (Nagelhus et al. 2004), and increased levels of glutamine synthetase, reciprocal alterations in synaptic activity might be directly reflected on the level of the tripartite synapse (Clarke and Barres 2013). As neurons and synapses share identical proteins (receptors, channels, cell adhesion molecules, ECM components and many regulatory proteins) in particular with astrocytes, it is an unresolved question at this point, in which cell type the observed changes in these ‘shared’ proteins upon auditory discrimination do really occur.

Protein phosphorylation is a key event in regulating activity, subcellular localization, and protein interactions at the synapse. Being a very rapid protein modification occurring already within seconds to minutes after stimulation, its duration depends on a delicate balance between protein kinases and phosphatases. Experience-dependent changes in phosphorylation capacities are known for decades (Ehrlich et al. 1977). Here, we found specific changes in phosphoprotein patterns 24 h after FMTD training, pointing to long-term deviations of the synaptic phosphoproteome. In the FC, a particularly prominent increase in phosphorylation was monitored for the non-receptor tyrosine kinase Src at Ser-17. This residue is known to be phosphorylated by protein kinase A in response to cAMP stimulation, e.g. by neurotransmitters or growth factors, where it may be involved in the control of neurite outgrowth via Rap1 activation (Obara et al. 2004; Kennedy et al. 2005; Amata et al. 2014). Another protein that is highly phosphorylated in the FC after FMTD training is DLGAP3/SAPAP3 at Ser-712. Mutants affecting this protein display defects at corticostriatal synapses and compulsive disorder-like behavior (Welch et al. 2007; Wan et al. 2014). Long-term up-regulation of Ser-712 phosphorylation in DLGAP3/SAPAP3 has also been reported in the rodent hippocampus paralleling spatial memory retrieval in the Barnes maze (Li et al. 2014).

In silico analysis of the data acquired in this study revealed several biological processes and pathways as modulated after the FMTD learning experiment. Membrane transport processes, modulation of cell–cell junctions and the cell-cortical actin-based cytoskeleton, RNA processing, RNA transport, and protein synthesis were affected in all the four brain regions (Tables 1 and S6). However, within these pathways, different components were found to be regulated (Fig. 5; see also Figure S4). Other biological pathways seemed to be modulated significantly only in one or two of the analyzed brain areas. This suggests spatial and/or temporal selectivity for the dynamics of synaptic proteomes, potentially representing differential processes of synaptic plasticity in the different brain regions at the investigated time point after the learning experiment.

Implications for understanding cortex-dependent memory formation during FMTD learning

Our previous studies in gerbils suggested that increased cortical glutamate and dopamine signaling during and immediately after FMTD learning may induce different protein synthesis-dependent processes that support initial memory retention for about 1 day as well as long-term memory consolidation and anterograde memory formation (Kraus et al. 2002; Tischmeyer et al. 2003; Schicknick and Tischmeyer 2006; Schicknick et al. 2008). Our present findings suggest that learning the new task and/or experiencing initial prerequisites necessary for learning, such as stimulus novelty, led to increased synaptic remodeling in brain regions implicated in FMTD learning and memory.

The vast majority of alterations detected in this study 24 h after the learning experiment were restricted to individual brain regions, implying that FMTD learning may induce region-specific synaptic proteome and phosphoproteome changes. In the HIP, SJ-enriched proteins representing the pathways ‘regulation of cellular mechanics by calpain protease’, ‘glutamate receptor signaling’, and ‘synaptic long-term potentiation’, a well-characterized cellular form of synaptic plasticity thought to underlie learning and memory, were regulated (Table 1). Regulation of the pathway ‘calcium signaling’ was detected in the FC, and ‘dopamine receptor signaling’ was monitored in striatal regions, which are the principal recipient structures of midbrain dopamine axons (Tritsch and Sabatini 2012). Memory consolidation is assumed to involve interactions of networks in multiple brain regions over days or weeks (Wiltgen et al. 2004; Frankland and Bontempi 2005). The HIP is believed to integrate information from distributed
cortical networks into a coherent memory trace and to mediate its temporary storage and retrieval. For remote memory storage and retrieval, neurons in the cortex, in particular in frontal regions, must undergo a tagging process early upon encoding (Lesburgueres et al. 2011); consolidation of the memory trace at the cortical level would then occur slowly via repeated reactivation of hippocampocortical networks. Corticostriall synaptic plasticity may enable the learned transformation of sound representation into behavioral action during auditory-cued decision-making (Xiong et al. 2015). Thus, in view of the concept of an active redistribution of memory representations from temporary into long-term stores (Squire and Alvarez 1995), the present findings may be indicative of learning-induced mechanisms mediating – probably under dopaminergic control (Reichenbach et al. 2015) – different aspects of memory consolidation. At the molecular level, it is tempting to speculate that mechanisms of post-translational protein modification, such as phosphorylation, calpain-activated proteolysis, and cytoskeletal reorganization, that have previously been implicated in long-term memory formation and maintenance (e.g. Routtenberg and Rekart 2005; Giese and Mizuno 2013; Kwapis and Helmstetter 2014), might participate in the management of FMTD memory for at least 1 day after the learning event.

In conclusion, this study revealed cross-regional as well as distinctive synaptic proteome and phosphoproteome changes in cortical and subcortical brain regions of mice 24 h after differential conditioning to FM tones, which may be indicative of mechanisms supporting different aspects of memory consolidation.

Acknowledgments and conflict of interest disclosure

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All experiments were conducted in compliance with the ARRIVE guidelines.

Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. (a) MIAPE-compliant MS-description for label-free MS proteomics. (b) MIAPE-compliant MSI-description for label-free MS proteomics. (c) MIAPE-compliant MS-description for MRM. (d) MIAPE-compliant MSI-description for MRM.

Table S2. Antibodies used for western blot verification.

Table S3. Proteins showing abundance changes in the SJ-enriched samples from different brain regions of mice 24 h after FMTD training.

Table S4. Phosphopeptides and deduced proteins showing abundance changes in the SJ-enriched samples from different brain regions of mice 24 h after FMTD training.

Table S5. Verification of the regulation of selected proteins.

Table S6. Data for single enrichment analysis with GeneCodis.

Figure S1. Performance in the FMTD paradigm.

Figure S2. Differential regulation of synaptic protein abundance in analyzed brain regions.

Figure S3. Verification of the regulation of selected proteins by western blot analysis.

Figure S4. Regional differences in the modulation of synaptic protein networks.

References


