Nascent Proteome Remodeling following Homeostatic Scaling at Hippocampal Synapses

Highlights
- Newly synthesized proteins were metabolically labeled during homeostatic scaling
- Approximately 6,000 newly synthesized proteins were detected using mass spectrometry
- Over 300 proteins were differentially regulated by synaptic up- and down-scaling
- Important synaptic proteins and disease-related proteins were regulated

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In Brief
Schanzenbächer et al. discover the newly synthesized proteome in response to the global activity manipulations that bring about homeostatic synaptic scaling. The synthesis of over 300 proteins was significantly differentially regulated by the induction of scaling, including neurotransmitter receptors, scaffolding, and signaling molecules.

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Nascent Proteome Remodeling following Homeostatic Scaling at Hippocampal Synapses

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SUMMARY

Homeostatic scaling adjusts the strength of synaptic connections up or down in response to large changes in input. To identify the landscape of proteomic changes that contribute to opposing forms of homeostatic plasticity, we examined the plasticity-induced changes in the newly synthesized proteome. Cultured rat hippocampal neurons underwent homeostatic up-scaling or down-scaling. We used BONCAT (bio-orthogonal non-canonical amino acid tagging) to metabolically label, capture, and identify newly synthesized proteins, detecting and analyzing 5,940 newly synthesized proteins using mass spectrometry and label-free quantitation. Neither up- nor down-scaling produced changes in the number of different proteins translated. Rather, up- and down-scaling elicited opposing transnational regulation of several molecular pathways, producing targeted adjustments in the proteome. We discovered ~300 differentially regulated proteins involved in neurite outgrowth, axon guidance, filopodia assembly, excitatory synapses, and glutamate receptor complexes. We also identified differentially regulated proteins that are associated with multiple diseases, including schizophrenia, epilepsy, and Parkinson’s disease.

INTRODUCTION

Neuronal networks are subject to fluctuations in both the magnitude and frequency of inputs, requiring plasticity mechanisms to stabilize network activity. Homeostatic synaptic scaling adjusts the strength of neuronal connections up or down in response to changes in input (Davis, 2013; Turrigiano, 2012). At the Drosophila neuromuscular junction, genetic manipulations that alter postsynaptic receptor function result in compensatory changes in presynaptic neurotransmitter release (see Davis, 2013 for review). At mammalian synapses, blocking action potential-mediated activity either pharmacologically (O’Brien et al., 1998; Turrigiano et al., 1998) by occluding sensory input (Desai et al., 2002) or by suppressing excitability (Burrone et al., 2002) can lead to a global up-scaling of synaptic responses. Conversely, a global stimulation of activity, effected by blocking GABA<sub>A</sub> receptor-mediated inhibition, leads to a significant down-scaling of synaptic responses (O’Brien et al., 1998; Turrigiano et al., 1998).

What is the nature of the molecular changes that bring about the synaptic scaling at mammalian synapses? Several groups have postulated that fluctuations in intracellular Ca<sup>2+</sup> levels initially “sense” changes in activity (Ibata et al., 2008; Thiagarajan et al., 2005) that may be read out by changes in the activation of CaMKIV resulting in changes in gene expression (Ibata et al., 2008). In addition, the expression of scaling up is mediated by the accumulation of GluR2-containing receptors (Gainey et al., 2009, 2015), likely driven by alterations with the glutamate receptor-interacting protein-1, GRIP-1 (Gainey et al., 2015), and protein-interacting-with C Kinase 1, PICK1 (Anggono et al., 2011). Other molecules implicated in various aspects of synaptic scaling include the neurotrophin brain-derived neurotrophic factor (BDNF) (Rutherford et al., 1998), the immediate early gene Arc (Shepherd et al., 2006), the cytokine TNF<sub>a</sub> (Steinmetz and Turrigiano, 2010; Stellwagen and Malenka, 2008), the immune molecule MHC1 (Goddard et al., 2007), β3 integrins (Cingolani et al., 2008), and the scaffold proteins PSD-95 and PSD-93 (Sun and Turrigiano, 2011). All of the above molecules were discovered using a candidate-based approach, making good “guesses” about the players that might be important, leaving open the possibility that many important molecules have not yet been discovered.

The long time course (~24 hr) required for most forms of synaptic homeostasis to be established (O’Brien et al., 1998; Turrigiano et al., 1998) suggests a requirement for changes in gene expression, either by regulated transcription or translation, or both. Indeed, upscaling elicited by a 24 hr TTX treatment is blocked by a transcription inhibitor (Ibata et al., 2008). In a form of homeostatic depression elicited by ChR2-stimulation of individual neurons, a requirement for both transcription and translation has been reported (Goold and Nicoll, 2010). Whether the classic homeostatic plasticity, elicited pharmacologically, requires changes in new protein translation has, surprisingly, not been addressed.

What is known about the transcriptomic and proteomic alterations that underlie homeostatic plasticity? A recent study...
studied a few selected transcripts with qPCR and showed that homeostatic upscaling resulted in a significant increase in the Gria1, Tet1, and Gad1 45b transcripts (Meadows et al., 2015). While alterations in transcripts can lead to changes in protein abundance, several studies have shown that in complex systems the correlation between the transcriptome and the proteome can be low (Gygi et al., 1999; Komatsu and Hossain, 2013; Jovanovic et al., 2015). As proteins are the primary and most common effectors in all cellular processes, it is important to determine the proteome directly to understand how plasticity fundamentally alters the neuronal and synaptic landscape.

Recent advances in proteomic technology have led to unprecedented coverage and depth in the identification of proteins in cells and tissues. A recent study, for example, identified over 8,000 proteins in each of several different brain areas, including the hippocampus (Sharma et al., 2015). In addition, over 2,000 proteins reliably associated with the postsynaptic density have also been identified (Distler et al., 2014; Peng et al., 2004; Pielot et al., 2012; Cajigas et al., 2012). Quantitative proteomic analyses that focus on the entire (unlabeled) proteome, however, do not allow one to identify proteins synthesized in response to plasticity. For example, if one observes an increase in protein levels, it is impossible to determine whether the increase is due to enhanced protein synthesis or decreased protein degradation. In order to isolate the analysis of the newly synthesized proteome associated with plasticity, metabolic labeling is required. Bio-orthogonal non-canonical amino acid tagging (BONCAT) enables pulsed metabolic labeling of newly synthesized proteins using non-canonical amino acids like azidohomoalanine (AHA) together with click-chemistry followed by mass spectrometry (Dieterich et al., 2006, 2007). Using this method, alterations in newly synthesized proteins can be directly determined. This approach has been applied to identify newly synthesized proteins in many systems (Landgraf et al., 2015), including in the hippocampus following treatment with dopamine (Hodas et al., 2012) or BDNF (Bowling et al., 2016).

The opposite phenotypes elicited during synaptic up-scaling and down-scaling represent a unique opportunity to examine the underlying proteomic plasticity and identify pathways that are bi-directionally or coordinately regulated. Here we detect an unprecedented number of newly synthesized proteins and provide a comprehensive map of the newly synthesized proteome following both up- and down-scaling.

RESULTS

To ascertain, first, whether pharmacologically elicited synaptic scaling requires protein synthesis, we conducted experiments in which protein synthesis was inhibited during the scaling manipulation (Figure S1). In order to elicit up-scaling, cultured hippocampal neurons were treated with the Na+ channel antagonist tetrodotoxin (1 μM) for 24 hr to elicit an increase in miniature excitatory postsynaptic current (mEPSC) amplitude (Figure S1A). The co-application of the protein synthesis inhibitor anisomycin (40 μM) prevented the mEPSC amplitude increase (Figure S1B). In order to elicit down-scaling, cultured hippocampal neurons (DIV 21) were treated with the GABA_A receptor antagonist bicuculline (40 μM) for 24 hr to elicit a decrease in mEPSC amplitude (Figure S1A). The co-application of the protein synthesis inhibitor anisomycin (40 μM) prevented the mEPSC amplitude decrease, indicating a requirement for new protein synthesis (Figure S1C). Taken together, these data demonstrate that protein synthesis is required for both up- and down-scaling elicited pharmacologically in cultured neurons.

In order to identify the specific proteins synthesized following global manipulations of neuronal activity, we used BONCAT (Figures 1 and 2; Dieterich et al., 2006) together with the paradigm of homeostatic scaling. For the duration of the scaling induction (24 hr of either bicuculline or TTX, as above) cultured hippocampal neurons (DIV 21) were treated with the non-canonical amino acid azidohomoalanine (AHA; 4 mM) to label the newly synthesized proteins associated with homeostatic scaling. A control group was exposed to AHA for 24 hr in the absence of either bicuculline or TTX. An additional negative control group was treated with methionine, the amino acid from which AHA is derived (Met; 4 mM) (Figure 2A). Following scaling induction, neuronal lysates were prepared and BONCAT (see Experimental Procedures) was performed to capture, purify, and identify the newly synthesized proteins (Figure 2A). For each set of experiments, we conducted five independent biological replicates that were each injected in four technical replicate LC-MS/MS runs (see Experimental Procedures; Figure 1 and S2). To identify proteins, we required a minimum of one tryptic peptide per protein (see Experimental Procedures; Table S9). With a few exceptions (see Experimental Procedures), any protein detected in a methionine control experiment was subtracted from our experimental dataset, and only proteins present in more than two biological replicates were retained for subsequent analysis.

In the above experiments, we obtained an unprecedented coverage and depth of the newly synthesized proteome. Our master dataset, comprising the union of the untreated, scaled-up, and scaled-down groups, includes 5,940 unique protein groups representing all key cellular processes, including metabolic enzymes, cell signaling, and structural proteins (Figures 1 and 2B; Table S1). We were able to detect most of the important neuronal membrane proteins including ion channels and a majority of the subunits of the excitatory and inhibitory neurotransmitter receptors, including AMPA and NMDA receptor subunits as well as GABA_A and GABA_B receptors (Figures 5 and S5; Tables S1 and S3). In addition, we identified many membrane proteins associated with presynaptic membranes and vesicles, including both vesicular and plasma membrane transporters (Figure 6; Tables S1 and S3). These data are described in much greater detail below.

In order to assess the depth and breadth of our newly synthesized proteome, we compared the newly synthesized proteome of control (not subjected to scaling) AHA-treated neurons to a global hippocampal proteome dataset (Sharma et al., 2015) (see Experimental Procedures; Figure 1). We found that there was substantial overlap between the pulse (24 hr)-labeled nascent hippocampal proteome and the global hippocampal proteome (Figure 2C). The AHA-labeled proteome also captured many proteins not identified in the global proteome, likely owing to the specific enrichment and reduced sample complexity of the AHA-proteome and the resulting expanded dynamic range. We considered the possibility that some of the AHA-exclusive
Figure 1. Overall Workflow Including Sample Structure, Preparation, Analyses and Data Processing, and Evaluation

Flow chart explaining the datasets used, how the data were filtered, and how we calculated the numbers of proteins in each figure as well as the conversion to genes, when appropriate.
Figure 2. Workflow and Characterization of the Newly Synthesized Proteome

(A) Workflow of experiments. Cultured hippocampal neurons (DIV 21) were treated with AHA (4 mM) and either vehicle, TTX (1 μM), or bicuculline (20 μM) for 24 hr. Control cultured neurons were exposed to methionine (4 mM) for 24 hr. Neurons were then harvested, and proteins were solubilized and clicked to alkyne-bearing beads. An on-resin digestion was performed, and peptides were then identified via liquid chromatography-mass spectrometry (LC-MS/MS). Proteins were identified and quantified using MaxQuant and other tools (see Experimental Procedures).

(B) Representation of protein groups in different cellular compartments.

(C) Venn diagram of the global hippocampal proteome (identified in Sharma et al., 2015) compared to the newly synthesized proteome (identified by 24 hr of AHA labeling).

(D) Plot of protein half-lives for the global versus newly synthesized proteomes. Available data from Cohen et al. (2013) for 60 AHA-labeled and global proteome proteins are shown. There is a trend for shorter half-lives of the AHA-identified proteins.

(E) Graph indicating the number of identified proteins across biological replicates for each group. Homeostatic scaling does not change the size of the nascent proteome. A significantly smaller number of proteins were identified in the negative control (methionine-treated) group (p < 0.0001), but none of the other groups differed significantly from one another. Error bars represent ± SEM.

(F) Reproducibility of protein identifications across biological replicates within each group.
proteins represent short-lived proteins that are less likely to be detected in the large global proteome sample. To examine this possibility, we compared the reported protein half-lives for the AHA-exclusive proteins to a comparable number of proteins from the global hippocampal proteome. Using data from Cohen et al. (2013), we extracted all of the half-life data for AHA-exclusive proteins analyzed in their dataset (n = 60) and compared the distribution of half-lives to the same number of proteins subsampled from the global hippocampal proteome. We observed a shift toward a shorter half-life in the distribution for AHA-labeled proteins (Figure 2D), suggesting that one feature of the AHA-exclusive proteins may be a faster turnover, making the proteins more difficult to capture and detect in global proteomic studies.

We next analyzed the newly synthesized proteome in control (untreated), scaled-up, and scaled-down samples. First we examined the total nascent proteome size and discovered that homeostatic scaling does not significantly alter the number of unique newly synthesized proteins in the total proteome. The total nascent proteome size in each group (control, scaled-up, and scaled-down) was between 5,100 and 5,200 proteins (Figures 1 and 2E; Table S1). In the negative control methionine-treated samples, there were ~594 proteins detected. Notably, in each AHA-treated group, over ~80% of the proteins identified were in 3/5 biological replicates and over ~90% of the proteins were identified in 2/5 biological replicates (Figure 2F). As there was no significant difference in the newly synthesized proteome size between groups, these data indicate that homeostatic scaling is associated with a balanced stimulation and inhibition of synthesis of protein groups.

We hypothesized that the dominant class of proteomic change during scaling would comprise changes in the abundance of proteins that perform essential cellular and synaptic functions. To analyze this, we compared protein abundances in the up-scaled or down-scaled nascent proteomes to control nascent proteomes using a label-free quantitation (LFQ)-based analysis (see Experimental Procedures; Figure 1). Altogether, we discovered over 300 proteins with significantly regulated expression levels following homeostatic plasticity (Figures 3A and S4; Table S2).

Figure 3. The Synthesis of ~300 Proteins Is Differentially Regulated by Homeostatic Scaling
(A) Scatterplot indicating every analyzed protein (n = 4,186). Proteins that were significantly regulated by homeostatic plasticity are indicated as mauve dots; all other proteins are gray. Histograms on the horizontal (teal) and vertical (orange) axes indicate the number of proteins that exhibited a given fold-change on the x (teal) and y (orange) axes.
(B) Venn diagram depicting the total number and low-abundant, newly synthesized proteins identified in each of the following groups: scaled-up (TTX-treated), scaled-down (bicuculline-treated), or untreated (control). Proteins were manually extracted as outlined in Experimental Procedures.
(C) Functional groups of significantly regulated proteins observed following up- or down-scaling.
enrichment of protein families mediating inositol-1,4,5-triphosphate-sensitive calcium release, sphingomyelin degradation, and cell-cell adhesion essential for dendritic spine morphogenesis as well as other cellular functions (Table S3). Taken together, these two groups of proteins exhibit consistent enhanced or reduced synthesis, regardless of the sign of the plasticity, and thus might be considered as “general” scaling proteins that respond to global changes in neuronal activity.

The remaining categories are represented by proteins significantly altered in a bi-directional manner: for these proteins, enhanced synthesis is associated with one form of plasticity (either up- or down-scaling) while reduced synthesis is associated with the other form (Figures 3A and 4; Table S2). When compared to control samples, there were 105 proteins that exhibited enhanced protein synthesis associated with up-scaling and reduced protein synthesis during down-scaling (Figure 3A, lower right quadrant). GO analysis of this group indicates a significant enrichment for proteins largely involved in synaptic function, including, for example, the family of Ca²⁺-calmodulin-dependent protein kinases (CamK) and proteins involved in neurotransmitter transport and exocytosis (Figure 4). Functional annotation analysis of this group indicates a significant enrichment for calcium-dependent protein serine/threonine phosphatases, splicing factors, transcription factors, calcineurin regulators, and synaptotagmins. Additional enriched proteins in this group are implicated in mRNA deadenylation, ubiquitin activation, cell growth, and other categories (Table S3). The above proteins should thus be considered as “specific” scaling proteins that respond with a specific signature to either homeostatic up- or down-scaling.

We also considered the entire population (~307) of regulated nascent proteins as a single group and examined, using a String analysis, the networks of regulated proteins and the interactions between them (Figure S3). We identified one large network of regulated proteins containing 95 network members with the number of interaction partners ranging from 1 to greater than 7. Protein hubs in this network were represented by several protein kinases including the Fyn kinase, Lyn kinase, Map kinase 3, and Protein kinase C beta. In addition, other proteins such as the FGF receptor were linked to several other significantly regulated proteins. The remaining networks comprising significantly regulated proteins were small, ranging from 2 to 6 proteins, and the remaining regulated proteins were not linked to networks.

Finally, we considered the possibility that elements of the homeostatic proteomic response could be newly synthesized proteins that were uniquely and exclusively associated with either the control or scaled groups. We found no highly abundant (>10 peptides) proteins exclusively in scaled-up or scaled-down neurons. As regulatory proteins may be low abundant or only yield a low number of detectable peptides, we manually inspected low-abundance proteins in our dataset based on reproducibility, spectral counting, and normalized peptide intensity ratios (for a detailed description, see Experimental Procedures). Acknowledging that this approach yields only trends rather than precise quantitative values, we identified 27 and 7 proteins with higher abundance in scaled-up or scaled-down neurons, respectively (Figures 1 and 3B; Tables S1 and S2). Using these “relaxed” criteria, newly synthesized proteins enriched in scaled-down neurons were implicated in glutamatergic synapses and excitability (Adcy2, Grin2a), calcium transport (Atp2b3), nerve growth factor signaling (Lmtk2), and other cellular functions (Table S2). The newly synthesized proteins enriched in scaled-down neurons included proteins involved in synapse formation (Arhgap) and modulation of dendritic spines (Dgk2) (Table S2). An additional six proteins were found to be enriched in both scaled groups (Table S2), including proteins involved in modulation of dendritic spine shape (Synpo), regulation of the number of excitatory synapses (C1ql3), and axonal branching (Fgf2).

We next determined the changes that occurred in different cell types, compartments, and signaling pathways. Within the postsynaptic compartment (Figure 5; Table S4), we found several examples where the opposite phenotypic endpoints of homeostatic scaling are associated with opposing effects on glutamate receptors. For example, some subunits of AMPA-type (Gria3, Grid1) and a kainate-type (Grik2) receptor showed a pattern of
upregulation during scaling-up accompanied in some cases (Grid1, Grik2) by downregulation during scaling-down, as expected if the receptors are modulated to increase or decrease sensitivity to neurotransmitter (Figure 5; Table S4). Unfortu-
nately, we did not obtain a sufficient number of unique peptides for Gria1 and 2 to conduct an analysis.) The NMDA receptor sub-
unit Grin2b exhibited a trend for downregulated synthesis in both up- and down-scaling, although to a much greater extent during
down-scaling (Figure 5). Other postsynaptic components that
erected regulation include Cript (Niethammer et al., 1998)
and Lrcc7 (Densin-180). Map kinase 3 (Mapk3), a kinase impli-
cated in long-term potentiation (LTP; Mazzucchelli et al., 2002)
and implicated in autism spectrum disorders (Kumar et al.,
2008), was significantly differentially regulated: enhanced in
scaled-up conditions and reduced in scaled-down conditions
(Figures 4 and 6). Some proteins in the Ap-3 family (Ap3b2 and Ap3m2), a heterotetrameric vesicle-coat protein complex that is important for protein sorting and clathrin recruitment, exhibited significantly different expression with up- and down-scaling and were significantly upregulated with up-scaling (Figures 4 and 6; Tables S2 and S4). Related to the actin cytoskeleton, several proteins (Evi, Sptbn4, and Vasp) showed extremely robust and significant regulation with down-scaling and also with up-scaling (Figure 6; Tables S2 and S4).

As homeostatic plasticity is known to involve regulation
of inhibitory transmission (Hartman et al., 2006; Kilman et al.,
2002; Maffei et al., 2004), we next examined the protein compo-
nents of GABAergic synaptic transmission that exhibit altered
protein synthesis (Figure S5). The family of GABA receptors
was significantly regulated and exhibited, in general, opposite
clearance of adhesion is required for structural plasticity
at synapses (Mayford et al., 1992; Schuster et al., 1996).
Nlgn2, in contrast, was significantly upregulated by homeostatic
up-scaling (Figure 5; Tables S2 and S4).

Within the functional area of vesicular trafficking and neuro-
transmitter release, we observed many significantly regulated
proteins (Figure 6; Table S4). Some vesicle-associated proteins
like members of the syntaxin family (Sy6 and Esyt1) exhibited
enhanced synthesis following down-scaling, whereas Snap29,
a protein involved in multiple membrane trafficking steps, and
Brsk1, a protein implicated in neurotransmitter release, were
significantly differentially regulated by homeostatic scaling and
reduced following down-scaling (Figures 4 and 6). Some pro-
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and clathrin recruitment, exhibited significantly different expres-
sion with up- and down-scaling and were significantly upregu-
late-scaling and reduced in scaled-down conditions
(Table S2 and S4). Several adhesion molecules, Tnr, Cdh11,
Pcdhc3, and Sema6d, were also significantly regulated with a
clear trend for downregulation in both forms of scaling (Figure 5;
Table S2 and S4), reminiscent of earlier studies showing that
downregulation of adhesion is required for structural plasticity
at synapses (Mayford et al., 1992; Schuster et al., 1996).
Nlgn2, in contrast, was significantly upregulated by homeostatic
up-scaling (Figure 5; Tables S2 and S4).

Within the functional area of vesicular trafficking and neuro-
transmitter release, we observed many significantly regulated
proteins (Figure 6; Table S4). Some vesicle-associated proteins

upregulation following up-scaling (Figure S5). Synthesis of the vesicular GABA transporter, Slc32a1, was reduced in down-scaled neurons and slightly enhanced in up-scaled neurons (Figure S5). Interestingly, Kif5a, a microtubule-dependent motor essential for the transport of GABA receptors (Nakajima et al., 2012) was significantly differentially regulated and upregulated in scaled-up neurons (Figure S5).

Since AHA-labeled newly synthesized proteins can originate from either the glial or neuronal cells present in our hippocampal cultures, we used bioinformatics (GeneAnalytics; see Experimental Procedures) to separately analyze the proteins associated with glial function. For the 5,940 proteins in the global AHA-labeled proteome, there are 6,339 associated genes; of these, 929 genes (14.7%) are associated with glial function (Table S6). For the 307 significantly regulated proteins in the AHA-proteome, there are 325 associated genes; of these, 41 genes (12.6%) are associated with glial function (Table S6). It is important to note that the GeneAnalytics tool we used identifies...
glial-associated genes, but does not identify glial-exclusive genes. As such, some of the regulated genes are shared with neurons, and the cell type where the protein is significantly altered—glia, neurons, or both—is not certain. Cell-type-specific labeling techniques (see Discussion) will certainly be useful to address this issue in the future.

As Ca^{2+}-regulated and synapse-to-nucleus signaling play a prominent role in all forms of plasticity, including homeostatic plasticity, we also searched for altered protein synthesis in these pathways (Figure 7; Table S4). The CamKII family of subunits, for example, showed a consistent trend of upregulated synthesis following scaling-up and downregulated synthesis following scaling-down (Figure 7; Table S2). A similar pattern was observed for the beta subunit of Protein kinase C (Prkcb) and several of the Map kinases (Mapk3, Map4k, and Mapk8) (Figure 7). The inositol trisphosphate receptor 2 (ITPR2) was also potently and significantly downregulated with both forms of plasticity, whereas the ITPR1 molecule was significantly downregulated exclusively with scaling-up (Figure 7). The family of Arhgap, negative regulators of Rho GTPases, together with the positive regulator of Rho GTPases and dendritic morphology regulator Tiam1 (Um et al., 2014), represent a hotspot for significant regulation during homeostatic scaling.

In the above sections, we highlighted some proteins that are altered during synaptic scaling. As defects in synaptic scaling have been proposed to contribute to diseases such as...
Alzheimer’s disease (Pratt et al., 2011) and epilepsy (Trasande and Ramirez, 2007), we asked whether any of the regulated proteins identified in this study are associated with neurodevelopmental, psychiatric, or neurodegenerative disorders. Forty-five percent of the proteins regulated during homeostatic scaling had an association with a neural disease. As shown in Figure 8, we queried regulated proteins associated with Alzheimer’s disease, Parkinson’s disease, Huntington’s disease, schizophrenia, epilepsy, autism, amyotrophic lateral sclerosis, multiple sclerosis, bipolar disorder, depression, and frontotemporal dementia (see also Table S7) and found over 166 proteins that were associated with at least one of the above diseases. Within this set of disease-related regulated proteins, we identified that, for example, Presenilin-1 (Psen), previously reported to be essential for homeostatic scaling (Pratt et al., 2011), is significantly regulated in both up- and down-scaling (Figures 6 and 8; Table S2).

We additionally examined the association of our regulated proteins with disease-related single-nucleotide polymorphisms identified in genome-wide association studies (GWAS) (Table S7). Here we again found that a large number (~95) of the proteins regulated by synaptic homeostasis are associated with neurological dysfunction.

**DISCUSSION**

In this study, we examined the proteomic changes that underlie homeostatic scaling in primary hippocampal neurons. We demonstrate for the first time that both up- and down-scaling elicited by pharmacological manipulation of neuronal activity require new protein synthesis. Using BONCAT (Dieterich et al., 2006), we selectively labeled, purified, and analyzed the newly synthesized neuronal proteomes. Here, BONCAT enabled us to track and visualize proteome adaptations during homeostatic scaling that are usually extremely challenging to examine in tissue samples due to both the dynamic range of the proteome and the relatively small degree of differential regulation. We identified over 5,000 proteins in each experimental group as well as the control group. Homeostatic scaling did not alter the absolute number of distinct newly synthesized proteins, but rather resulted in differences in which proteins were synthesized and to what degree. We detected ~300 proteins that exhibited significantly altered protein synthesis as a result of the global activity manipulations that induce homeostatic scaling. Recently, other groups have combined BONCAT with SILAC in order to include an additional validation step for new protein synthesis and to enable isotope-based, robust quantification (Bagert et al., 2014; Eichelbaum and Krijgsveld, 2014). Here we circumvented the need for SILAC, applying stringent control experiments and analyses and a highly stable LC-MS/MS setup, producing the largest dataset for the newly synthesized neuronal proteomes to date. We also investigated “low-abundance” proteins that are notoriously challenging to quantify due to the limited number of detectable peptides or low peptide intensities. We employed a combination of reproductibility, spectral counting, and normalized peptide intensities to manually evaluate a subset of low-abundance proteins, and as such these evaluations are “semi-quantitative”; the derived lists represent “trends” that may be of interest.

The significantly regulated newly synthesized proteins we identified span many functional categories and reside in different cellular compartments. Because of the time course of the plasticity and the duration of the metabolic labeling (24 hr), these proteins can represent regulated “sensors,” which respond to the activity manipulation, or “effectors,” which drive the regulatory processes.
implementation of the homeostatic responses. In addition to these proteins, we explicitly evaluated the previously published data documenting roles for various proteins in homeostatic scaling (Table S5). Of the 29 scaling-related proteins we identified in the literature, we detected 20 as newly synthesized in our dataset (∼69%). Of these 20 proteins, we detected either a trend for regulation or significant regulation associated with scaling for 65% percent. We also queried the extent to which our significantly regulated proteins have been associated with various neurological diseases and found that a large fraction (166 genes, ∼51%) have been implicated in one or more neurological diseases. An emerging view that incorporates the profound cumulative understanding that many neurological diseases are associated with mutations in hundreds to thousands of proteins is the notion that a loss of homeostasis at the level of neural circuits is causal (Ramocki and Zoghbi, 2008). The number of proteins identified and regulated by homeostatic scaling here provides a starting point to examine how dysregulation of these “homeostatic” proteins contributes to a variety of neuronal disorders.

Are the two forms of plasticity, homeostatic up-scaling and down-scaling, on a single mechanistic continuum, or are orthogonal mechanisms used? Most mechanistic studies have focused on up-scaling, examining the role of molecules that are associated with AMPA receptor trafficking or stabilization. A general question is whether the same mechanisms run in reverse for down-scaling. We observed multiple protein families and regulatory pathways that exhibited opposite regulation depending on the sign of the scaling (Figures 4 and S3), suggesting that these two forms of plasticity can be mediated by differential regulation of a common molecular-protein continuum. These protein families include, for example, the ionotropic glutamate receptor complex, proteins associated with neurotransmitter transport and exocytosis, and transcription factors. These data support the idea that at least some of the opposite phenotypes elicited by up- and down-scaling are driven mechanistically by an overlapping cast of molecular players, regulated in different directions. If the global manipulations of activity used here and in other studies possess a common sensor (e.g., Ca^{2+}), then the downstream mechanisms that bring about scaling-up or scaling-down could activate Ca^{2+} sensors with different sensitivities (e.g., Sutton et al., 2007) to bring about a concerted protein synthesis response.

A central question for all forms of cellular plasticity, of course, is the degree to which phenotypic changes are driven by changes in transcription and/or translation. A previous study indicated a requirement for transcription in homeostatic up-scaling (Ibata et al., 2008). Here we show for the first time that inhibition of protein synthesis during the initial 12 hr of scaling induction blocks both scaling-up and scaling-down. The period of protein synthesis inhibition was brief relative to the half-life of most neuronal/synaptic proteins (median = ∼3.5 days; Cohen et al., 2013), and there was no effect on baseline mEPSC frequency or amplitude. We thus conclude that the observed inhibition of scaling reflects a specific requirement for newly synthesized proteins in the expression of homeostatic scaling. Whether the observed changes in protein synthesis are due to changes in translation specifically or accompanied by changes in transcript level is an interesting question. In our data, there are several examples where multiple elements of a common signaling cascade are regulated (e.g., Figure 7). An open issue concerns the transcriptional or translational regulatory mechanisms that coordinate the changes in neuronal compartments so that elements in the same signaling pathway are consistently regulated to bring about change at the end-point, e.g., a positive or negative modulation of synaptic strength. In addition, although it has not been explicitly addressed here, it is obvious that the regulated degradation of proteins must also play a role in sculpting the proteome during homeostatic scaling. Indeed, we observed many proteins in the ubiquitin proteasome pathway that were regulated by scaling (Figure 3C; Table S3).

In addition to the proteomic changes described here, protein degradation, protein modifications, and/or transient protein complex formation play critical roles in adjusting synaptic strength. Indeed, Ehlers (2003) identified a cohort of synaptic proteins that were differentially regulated by activity manipulations and noted that inhibition of the ubiquitin proteasome pathway blocked the differential regulation. In addition, fast adaptions, such as short-term potentiation or depression, often rely heavily on modifications of existing proteins. In support of this idea, “kinases/phosphatases” represent the largest differentially regulated group in our dataset (Figure 3C; Table S3). For example, we found multiple MAP kinases and associated proteins (Table S3) that play a central role in multiple cellular processes. Also, our String analysis revealed that the tyrosine-protein kinases Lyn and Fyn represent central interaction “hubs” of the differentially regulated proteins in our dataset. Accordingly, future studies have to focus on obtaining complementary data on post-translational modifications and transient complex formation during homeostatic scaling.

We used BONCAT in hippocampal cultures to discover the nascent proteomic response to both up- and down-scaling. As the non-canonical amino acid AHA can be charged by the wild-type MetRS, all cells present in the culture can, in theory, contribute to the newly synthesized proteins we analyzed. Using bioinformatics, we were able to approximate attributions of some proteins to glia or interneurons, but this type of analysis is clearly limited. For example, it has been shown that homeostatic scaling can also be elicited at inhibitory synapses (Hartman et al., 2006). As such, common proteins in excitatory and inhibitory neurons that exhibit regulation in opposite directions might “cancel each other out” and hence not be detected as significantly regulated in our study. To gain selective access to particular cell classes, a recent development in which a mutant MetRS enables cell-type-specific labeling could be used in future experiments. This has already been employed in both C. elegans (Yuet et al., 2015) and Drosophila (Erdmann et al., 2015) to monitor protein synthesis in specific cell types. Applying this technology to homeostatic scaling and other forms of plasticity will allow us to discern the coordinated proteomic response in the different cell types that make up neuronal circuits.

**EXPERIMENTAL PROCEDURES**

**Preparation and Maintenance of Cultured Neurons**

Dissociated hippocampal neurons were prepared and maintained as previously described (Aakalu et al., 2001). Briefly, hippocampi from postnatal day
0–2 rat pups (strain Sprague–Dawley) were dissected out and dissociated by either trypsin or papain and plated at a density of 40,000 cells/cm² onto poly-D-lysine-coated glass-bottom Petri dishes (MatTek). Cultures were maintained in Neurobasal A medium containing B-27 and Glutamax supplements (Invitrogen) at 37°C for 21 days before experiments commenced. All experiments were carried out with the approval of the German animal experiment authorities.

**Electrophysiology**

Whole-cell recordings were performed in hippocampal culture neurons (DIV 21–27, 40 K cell density) held at ~70 mV in voltage clamp mode. To induce homeostatic plasticity, neurons were pretreated with either bicuculline (40 μM) or TTX (2 μM) for 24 hr. To block protein synthesis, neurons were incubated in anisomycin (40 mM) for the first 12 hr of the treatment. mEPSCs were recorded in hippocampal culture neurons (DIV 21–27, 40 K cell density) held at 37°C for 21 days before experiments commenced. All experiments were carried out with the approval of the German animal experiment authorities.

**Sample Preparation and MS Analyses**

Dissociated hippocampal neurons (DIV 21, two dishes per condition, ~800,000 cells in total) were incubated with 4 mM AHA (or methionine for the control group) and treated with bicuculline (20 μM) or tetrodotoxin (1 μM) for 24 hr. After incubation, the cells were washed with cold DPBS containing protease inhibitor cocktails (Complete EDTA-free, Roche), harvested, snap-frozen in liquid nitrogen, and stored at ~80°C until purification.

For cell lysis, the samples (cell pellets) were centrifuged briefly, the pellets resuspended in lysis buffer (100 μL, 8 M urea, 200 mM Tris [pH 8.4], 4% CHAPS, 1 M NaCl, Complete EDTA-free protease inhibitor), and lysed using a pistol. After sonication (4 × 30 s at 4°C), benzonase (1 μL of a ≥250 units/μL stock solution) was added to the solubilized samples, incubated for 5 min, and centrifuged for 5 min at 10,000 × g. The supernatants (~200 μL) were subsequently incubated with freshly prepared catalyst solution (250 μL) and Alkyne-Sepharose slurry (50 μL; see manufacturer’s protocol, Thermo Fisher Scientific) and incubated for 19 hr in the dark under gentle agitation.

After a short centrifugation (2 min, 1,000 × g), the beads were washed twice (900 μL each) and incubated with SDS wash buffer (100 mM Tris [pH 8], 1% SDS, 250 mM NaCl, 5 mM EDTA) containing 10 mM TCEP (250 μL). After incubation for 45 min at 55°C under gentle agitation, the samples were centrifuged for 5 min at 10,000 × g and the supernatants discarded. Each sample was then incubated with 250 μL SDS wash buffer containing a final concentration of 95 mM iodoacetamide for 30 min at room temperature in the dark under gentle agitation.

The samples were then transferred into pre-washed (400 μL H2O, LC-MS/MS-ChromaSolv; 400 μL SDS wash buffer) Pierce Spin columns and washed with 20 mL SDS wash buffer, 20 mL 8 M urea in 100 mM Tris (pH 8), and 20 mL 20% (v/v) acetonitrile/water. After washing, 250 μL of digestion buffer (100 mM Tris, 2 mM CaCl2, 10% acetonitrile) was added to each column and the resin transferred to an Eppendorf tube. After one washing step with digestion buffer, ~0.65 μg of EndoLysC was added to each tube and incubated overnight (~22 hr) at 37°C with constant agitation. For the second digestion step, each sample was incubated with 0.65 μg trypsin at 37°C overnight (~22 hr) with constant agitation.

The proteolytic peptides were extracted by incubating the resin with 500 μL H2O (0.1% TFA), centrifuging for 5 min at 1,000 × g, and collecting the supernatant. This step was repeated, and the combined supernatants were desalted using cC18-SepPak columns (Waters Corp.). The SepPak resin was washed with 2 mL of 50% acetonitrile/water (5% acetic acid) and equilibrated using 2 mL water (0.1% TFA). Each sample (~1 mL) was loaded onto a column and washed with 2 mL of water (0.1% TFA) and 200 μL of water (0.5% acetic acid). Peptides were eluted using 0.5 mL of 50% acetonitrile/water (0.5% acetic acid), dried using a Speed-Vac, and stored at –80°C until LC-MS/MS analysis.

For LC-MS/MS injection, the dried peptide fractions were dissolved in 5% acetonitrile with 0.1% formic acid, and subsequently loaded using a nano-HPLC (Dionex U3000 RSLCnano) on reverse-phase columns (trapping column: particle size 3 μm, C18, L = 20 mm; analytical column: particle size < 2 μm, C18, L = 50 cm; PepMap, Dionex/Thermo Fisher Scientific). Peptides were eluted in gradients of water (buffer A: water with 5% [v/v] acetonitrile and 0.1% formic acid) and acetonitrile (buffer B: 20% [v/v] water, 80% [v/v] acetonitrile, and 0.1% formic acid). All LC-MS/MS-grade solvents were purchased from Fluka. Gradients were ramped from 4% to 48% B in 178 min at flowrates of 300 nL/min. Peptides eluting from the column were ionized online using a Thermo nanoFlex ESI source and analyzed in a Thermo “Q Exactive Plus” mass spectrometer. Mass spectra were acquired over the mass range 350–1,400 m/z, and sequence information was acquired by computer-controlled, data-dependent automated switching to MS/MS mode using collision energies based on mass and charge state of the candidate ions (TOP12, MS resolution 70 K, MS² resolution 35 k; injection time: 120 ms, full parameter set in Table S9). All samples were measured in quadruplicate LC-MS/MS runs.

**Bioinformatic Processing and Criteria**

Raw data were loaded into MaxQuant (version 1.5.3.8; Cox and Mann, 2008) and analyzed with a customized Andromeda LFO parameter set (see Table S9). In brief, spectra were matched to a Rattus norvegicus database downloaded from uniprot.org (34,170 entries, reviewed and non-reviewed) and a contaminant and decoy database. Precursor mass tolerance was set to 20 ppm, fragment ion tolerance to 4.5 ppm, with fixed modification of Cys residues (carboxyamidomethylation +57.021) and variable modifications of Met residues (Ox +15.995, Lys residues (Acetyl +42.011), Asn and Gln residues (Deamidation +0.984), and N termini (carbamidyltaion +43.006). Peptide identifications were calculated with FDR = 0.01, and proteins with one peptide per protein were included for subsequent analyses.

Five independent biological replicates were processed for each group (methionine control, untreated cells, bicuculline-treated cells, tetrodotoxin-treated cells). After subtraction of background proteins, protein lists for each group were compiled containing proteins identified in at least 2 out of 5 replicates. The methionine content of our AHA-labeled proteins was not different compared to a global proteome (Rattus norvegicus, Uniprot database) as, on average, 2.38 and 2.40 met residues are present per 100 amino acids, respectively.

For full details on the data processing, please see Supplemental Experimental Procedures.

**ACCESSION NUMBERS**

The proteomics data associated with this manuscript have been uploaded to the PRIDE online repository: PXD004889 (Vizcaino et al., 2016).

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, five figures, and nine tables and can be found with this article online at http://dx.doi.org/10.1016/j.neuron.2016.09.058.

**AUTHOR CONTRIBUTIONS**

C.T.S., J.D.L., and E.M.S. designed experiments; C.T.S., S.S., and J.D.L. conducted and analyzed experiments. E.M.S. wrote the manuscript; all authors contributed to writing and editing. All authors edited and revised the manuscript.

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Supplemental Information

Nascent Proteome Remodeling following Homeostatic Scaling at Hippocampal Synapses

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Schanzenbaecher et al., Supplemental Materials.

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Supplemental Table 5.
Supplemental Table 6.
Supplemental Table 7.
Supplemental Table 9.
Supplemental Fig. 1

A

Anisomycin

12 h 12 h

TTX / BIC

B

control

TTX

TTX + Aniso

C

control

BIC

BIC + Aniso

Peak Amplitude (pA)

ns

****

control TTX TTX + Aniso

Peak Amplitude (pA)

ns

control BIC BIC + Aniso
Supplemental Fig. 4

Log₂ fold change scaled-down / untreated

Log₂ fold change scaled-up / untreated

n=4186 proteins

unregulated proteins
regulated proteins
Supplemental Figures.

Supplemental Figure 1, related to Figure 2. Protein synthesis is required for homeostatic up- and down-scaling.

(A) Scheme indicating experimental work-flow. The protein synthesis inhibitor anisomycin (40 μM) was applied for the first 12 hrs of scaling induction (24 hrs) by either TTX (1 μM) or bicuculline (40 μM).

(B) Representative electrophysiological recordings of miniature excitatory postsynaptic currents (mEPSCs) from control, TTX and TTX + anisomycin experiments. Analysis of mEPSC amplitude for groups, as indicated. TTX-treated neurons exhibited significantly larger mEPSCs than either control (p <0.0001) or TTX + aniso-treated neurons (p< 0.001), which did not differ significantly from one another. There was no change in mEPSC frequency produced by TTX treatment (data not shown).

(C) Representative electrophysiological recordings of miniature excitatory postsynaptic currents (mEPSCs) from control, bicuculline and bicuculline + anisomycin experiments. Analysis of mEPSC amplitude for groups, as indicated. Bicuculline-treated neurons exhibited significantly smaller mEPSCs than either control (p <0.0066) or Bicuculline + aniso-treated neurons (p< 0.0023), which did not differ significantly from one another. There was no change in mEPSC frequency produced by Bicuculline treatment (data not shown).

Supplemental Figure 2, related to Figures 2 and 3. Reproducibility of the dataset.

(A) Technical replicates for the global sample showing the average reproducibility of the 4 technical replicates that make up each of the 5 biological replicates.

(B) Biological replicate reproducibility of significantly regulated proteins in each of the quadrants of Figure 4A. Quadrants are as indicated and colored labels indicate the condition, scaling-up (teal), scaling-down (yellow) and whether the quadrant represents enhanced (“up”) or reduced (“down”) synthesis.

(C) Protein groups and numbers of proteins represented in the 4 quadrants of Figure 4A.
Supplemental Figure 3, related to Figure 4. Network analysis of all differentially regulated proteins. String pathway analysis showing that many (95) of the regulated proteins can be assembled into a single, large interactive network.

Supplemental Figure 4, related to Figure 4. Scatterplot of significantly regulated proteins showing quadrant localization with labels on the proteins that exhibit high levels of regulation.

Supplemental Figure 5, related to Figures 5 and 6. Proteins associated with inhibitory synaptic transmission regulated by homeostatic scaling. Scheme of an inhibitory synapse. Proteins in grey boxes were detected in our samples, call-out boxes are included for all proteins which fulfilled the criteria for the display of intensity values (see methods). The bars, teal and ochre, represent the regulation of the indicate protein in up- and down-scaling, respectively. Asterisks within the colored bar indicate that the protein exhibited significant (ANOVA FDR < 0.05 and Fisher LSD post-hoc p < 0.05) regulation relative to control; asterisks associated with the black lines indicate the protein exhibited significant (ANOVA FDR < 0.05 and Fisher LSD post-hoc p < 0.05) regulation between up- and down-scaling.
Supplemental Tables.

Supplemental Table 1 (separate file), related to Figure 2. Table of all proteins identified in each group.

Supplemental Table 2a, (separate file), related to Figure 3. Contains all 307 significantly regulated proteins.

Supplementary Table 2b, related to Figure 3. Low-abundance proteins with trends for enrichment conditions.

Supplemental Table 3, (separate file), related to Figure 3. Identified and significantly regulated proteins according to functional groups.

On different pages (tabs) we list the following protein groups:
   a) Ion channels and ion transport
   b) Cytoskeletal, cell adhesion and motor proteins
   c) Synapse and cell junction
   d) Kinases and phosphatases
   e) Neurotransmitters and Exocytosis
   f) Gene transcription
   g) mRNA regulation, protein translation and protein degradation

Supplemental Table 4, (separate file), related to Figures 5-7. All proteins shown in Figures 5-7 with corresponding analysis.

Supplemental Table 5, (separate file), related to Figures 3 and 4. List of previously implicated homeostasis molecules and their detection and regulation in this study.

Supplemental Table 6, (separate file), related to Figures 5-7. Newly synthesized significantly regulated proteins of potential glial origin.

Supplemental Table 7, (separate file), related to Figure 8. Newly synthesized significantly regulated proteins associated with some neurodegenerative, neuropsychiatric and neurodevelopmental disorders.
Supplemental Table 8, related to Figure 3. Full list of protein groups (<50 proteins) found using 1D annotation enrichment (see Materials and Methods). Proteins are sorted by first column (mean log₂ fold change Bic/TTX), table also includes Median log₂ fold change (Bic/TTX), group annotation name, group identifier, the number of proteins in the group, P value for 1D enrichment, Benjamini-Hochberg FDR and annotation database the group term derives from (GOCC = Gene Ontology Cellular Component, GOMF = Gene Ontology Molecular Function, GOBP = Gene Ontology Biological Processes, EMBL-EBI databases Pfam (Protein Families) and InterPro).

Supplemental Table 9, (separate file), related to Figure 1. Full list of LC-MS and MaxQuant parameters used in this study.
| Protein ID(s) | Gene symbol(s) | Peptide ratio Bic/Untd | Log2 LFQ Bic Untd TTX | TTX: number of biological replicates | Bic: number of biological replicates | Untd: number of biological replicates | Bic-Untd (±pooled standard error) | Bic/Untd (±pooled standard error) | Bic: mean | Bic: coefficient of variation | Untd: mean | Untd: coefficient of variation | Total number of peptides | Mol. weight [kDa] | Bioinformatics | Sequence length | Score |
|--------------|---------------|-----------------------|-----------------------|-------------------------------------|-------------------------------------|-------------------------------------|----------------------------------|----------------------------------|-----------------|-----------------------------|-----------------|-----------------------------|--------------------------|----------------|-------------------------|------------------|--------|
| Gpr158       |               | 0.24 0.43 0.3 -1.47±0.772 -0.29±0.915 -1.18±0.457 4 5 3 1.3 3.2 4.1 0.5 2.2 3.8 23.204 24.384 24.674 0.206 0.831 1.956 2 134.83 1206 76.474 |
| Adarb1       |               | 0.52 0.63 0.39 -0.106±0.252 0.786±0.204 -0.892±0.206 5 5 5 2.6 4.1 2.4 1.1 1.5 0.9 23.483 24.375 23.589 0.32 0.171 0.316 2 77.924 711 47.953 |
| Arvcf        |               | 0.49 0.62 0.37 0.631±0.162 1.062±0.177 -0.43±0.16 4 5 4 1.5 2.6 1.6 0.4 1.4 0.4 21.801 22.231 21.169 0.146 0.203 0.196 1 105.49 973 22.025 |
| Cacna2d3     |               | 0.47 0.6 0.38 -0.3±0.626 0.827±0.532 -1.127±0.51 2 4 3 1.1 1.9 1.3 0.2 1 0.4 21.558 22.685 21.858 0.35 0.55 0.72 2 121.94 1082 4.3118 |
| Grin2a       |               | 0 0.42 0 - - - 0 4 1 0 1.3 1.8 - 0.4 - - 22.826 - - 0.279 - 7 165.48 1464 5.0079 |
| Arhgap33     |               | 0.56 0.5 0.56 - - - 4 1 1 1.3 1 1 0.5 - - 22.994 - - 0.204 - - 1 137.39 1284 3.1981 |
| Gab2         |               | 0.6 0.5 0.6 0.288±0.287 0.201±0.28 0.087±0.302 4 4 5 1.5 1 1 0.4 0 0 21.722 21.634 21.434 0.31 0.132 0.277 2 73.327 665 3.4289 |
| Bnip3        |               | 0.6 0.63 0.47 0.489±0.549 0.595±0.467 -0.106±0.607 5 5 4 1.6 1.8 1.1 0.6 1.1 0.1 22.887 22.993 22.398 0.993 0.777 0.379 2 20.978 187 9.5344 |
| RGD1309748   |               | 1 - 1 - - - 3 0 0 1.1 0 0 0.1 - - - - - - - - 2 30.939 275 1.6071 |
| Gab2         |               | 0.36 0.47 0.38 - 0.217±0.235 - 4 5 3 1.1 1.8 2 0.3 0.9 0.6 - 22.366 22.149 - 0.228 0.289 4 387.61 3564 14.824 |
| Gpd1l        |               | 0.35 0.52 0.32 -0.484±0.292 0.465±0.23 -0.949±0.253 4 4 4 2.3 4.7 4.3 0.7 0.8 0.4 25.245 26.194 25.729 0.355 0.191 0.306 1 38.066 351 69.156 |
| Csmd1        |               | 0.36 0.47 0.38 - 0.217±0.235 - 4 5 3 1.1 1.8 2 0.3 0.9 0.6 - 22.366 22.149 - 0.228 0.289 4 387.61 3564 14.824 |
| Gab2         |               | 0.6 0.5 0.6 0.288±0.287 0.201±0.28 0.087±0.302 4 4 5 1.5 1 1 0.4 0 0 21.722 21.634 21.434 0.31 0.132 0.277 2 73.327 665 3.4289 |
| Gab2         |               | 0.6 0.5 0.6 0.288±0.287 0.201±0.28 0.087±0.302 4 4 5 1.5 1 1 0.4 0 0 21.722 21.634 21.434 0.31 0.132 0.277 2 73.327 665 3.4289 |
| Protein ID(s) | Gene symbol(s) | Peptide ratio | Bic-Untd (±pooled biological unique peptides) | Bic-TTX (±pooled biological unique peptides) |
|--------------|----------------|--------------|-----------------------------------------------|-----------------------------------------------|
| P36800, M0R9G2, Q5D178 | A2m, LOC100911545 | 0.48 0.37 0.61 | -0.298±0.253 -0.71±0.28 0.412±0.249 | 5 5 5 3.2 2 3.5 0.9 1.1 0.9 |
| D4AAE6 | Rab20 | 0.55 0.39 0.65 | 0.358±0.175 - | 5 4 4 2.1 1.1 1.7 0.7 0.2 0.6 |
| Usp11 | 0.5 0.37 0.62 | -0.401±0.255 -0.835±0.242 0.434±0.189 | 5 5 5 1.7 1.1 1.8 0.4 0.1 0.4 |
| D4AAE6 | Trip1 | 0.9 0.5 0.7 | 0.358±0.175 - | 5 4 4 2.1 1.1 1.7 0.7 0.2 0.6 |
| Q9R1K2-4, Q9R1K2-2, Q9R1K2, Q9R1K2-3, F1M939, A0A096P6L6 | | | | |
| Q9R1K2-4, Q9R1K2-2, Q9R1K2, Q9R1K2-3, F1M939, A0A096P6L6 | | | | |
| Q9R1K2-4, Q9R1K2-2, Q9R1K2, Q9R1K2-3, F1M939, A0A096P6L6 | | | | |
| Q9R1K2-4, Q9R1K2-2, Q9R1K2, Q9R1K2-3, F1M939, A0A096P6L6 | | | | |
| Q9R1K2-4, Q9R1K2-2, Q9R1K2, Q9R1K2-3, F1M939, A0A096P6L6 | | | | |
| Q9R1K2-4, Q9R1K2-2, Q9R1K2, Q9R1K2-3, F1M939, A0A096P6L6 | | | | |
| Q9R1K2-4, Q9R1K2-2, Q9R1K2, Q9R1K2-3, F1M939, A0A096P6L6 | | | | |
| Q9R1K2-4, Q9R1K2-2, Q9R1K2, Q9R1K2-3, F1M939, A0A096P6L6 | | | | |
| Q9R1K2-4, Q9R1K2-2, Q9R1K2, Q9R1K2-3, F1M939, A0A096P6L6 | | | | |
| Q9R1K2-4, Q9R1K2-2, Q9R1K2, Q9R1K2-3, F1M939, A0A096P6L6 | | | | |
| Q9R1K2-4, Q9R1K2-2, Q9R1K2, Q9R1K2-3, F1M939, A0A096P6L6 | | | | |
| Q9R1K2-4, Q9R1K2-2, Q9R1K2, Q9R1K2-3, F1M939, A0A096P6L6 | | | | |
| Q9R1K2-4, Q9R1K2-2, Q9R1K2, Q9R1K2-3, F1M939, A0A096P6L6 | | | | |
| Q9R1K2-4, Q9R1K2-2, Q9R1K2, Q9R1K2-3, F1M939, A0A096P6L6 | | | | |
| Q9R1K2-4, Q9R1K2-2, Q9R1K2, Q9R1K2-3, F1M939, A0A096P6L6 | | | | |
| Q9R1K2-4, Q9R1K2-2, Q9R1K2, Q9R1K2-3, F1M939, A0A096P6L6 | | | | |
| Q9R1K2-4, Q9R1K2-2, Q9R1K2, Q9R1K2-3, F1M939, A0A096P6L6 | | | | |
| Q9R1K2-4, Q9R1K2-2, Q9R1K2, Q9R1K2-3, F1M939, A0A096P6L6 | | | | |
| Q9R1K2-4, Q9R1K2-2, Q9R1K2, Q9R1K2-3, F1M939, A0A096P6L6 | | | | |
| Q9R1K2-4, Q9R1K2-2, Q9R1K2, Q9R1K2-3, F1M939, A0A096P6L6 | | | | |
| Q9R1K2-4, Q9R1K2-2, Q9R1K2, Q9R1K2-3, F1M939, A0A096P6L6 | | | | |
| Q9R1K2-4, Q9R1K2-2, Q9R1K2, Q9R1K2-3, F1M939, A0A096P6L6 | | | | |
| Q9R1K2-4, Q9R1K2-2, Q9R1K2, Q9R1K2-3, F1M939, A0A096P6L6 | | | | |
| Q9R1K2-4, Q9R1K2-2, Q9R1K2, Q9R1K2-3, F1M939, A0A096P6L6 | | | | |
| Q9R1K2-4, Q9R1K2-2, Q9R1K2, Q9R1K2-3, F1M939, A0A096P6L6 | | | | |
| Q9R1K2-4, Q9R1K2-2, Q9R1K2, Q9R1K2-3, F1M939, A0A096P6L6 | | | | |
| Q9R1K2-4, Q9R1K2-2, Q9R1K2, Q9R1K2-3, F1M939, A0A096P6L6 | | | | |
| Q9R1K2-4, Q9R1K2-2, Q9R1K2, Q9R1K2-3, F1M939, A0A096P6L6 | | | | |
| Q9R1K2-4, Q9R1K2-2, Q9R1K2, Q9R1K2-3, F1M939, A0A096P6L6 | | | | |
| Q9R1K2-4, Q9R1K2-2, Q9R1K2, Q9R1K2-3, F1M939, A0A096P6L6 | | | | |
| Q9R1K2-4, Q9R1K2-2, Q9R1K2, Q9R1K2-3, F1M939, A0A096P6L6 | | | | |
| Q9R1K2-4, Q9R1K2-2, Q9R1K2, Q9R1K2-3, F1M939, A0A096P6L6 | | | | |
| Q9R1K2-4, Q9R1K2-2, Q9R1K2, Q9R1K2-3, F1M939, A0A096P6L6 | | | | |
| Q9R1K2-4, Q9R1K2-2, Q9R1K2, Q9R1K2-3, F1M939, A0A096P6L6 | | | | |
| Q9R1K2-4, Q9R1K2-2, Q9R1K2, Q9R1K2-3, F1M939, A0A096P6L6 | | | | |
| Q9R1K2-4, Q9R1K2-2, Q9R1K2, Q9R1K2-3, F1M939, A0A096P6L6 | | | | |
| Q9R1K2-4, Q9R1K2-2, Q9R1K2, Q9R1K2-3, F1M939, A0A096P6L6 | | | | |
| Q9R1K2-4, Q9R1K2-2, Q9R1K2, Q9R1K2-3, F1M939, A0A096P6L6 | | | | |
| Q9R1K2-4, Q9R1K2-2, Q9R1K2, Q9R1K2-3, F1M939, A0A096P6L6 | | | | |
| Mean log, fold change | Median log, fold change | Bic/TTX | ic/TTX | Annotation name | Identifier | Annotation size | P value | Benj. Hoch. FDR | Annotation database |
|-----------------------|------------------------|---------|--------|----------------|------------|----------------|---------|---------------|-------------------|
| 0.04                  | 0.03                   | 0.14    | 0.11   | Exocytosis      | GO:0006877 | 46             | 2.30E-06 | 0.0001         | GOBP slim         |
| 0.25                  | 0.24                   | 0.32    | 0.24   | Exocytosis      | GO:0006877 | 40             | 2.20E-05 | 0.0005         | GOBP slim         |
| 0.12                  | 0.12                   | 0.22    | 0.22   | Penkaryon       | GO:0043204 | 32             | 3.90E-04 | 0.0029         | GOBP slim         |
| 0.33                  | 0.33                   | 0.35    | 0.35   | C2 domain       | PP0168     | 38             | 1.00E-05 | 0.0136         | GOBP slim         |
| 0.29                  | 0.29                   | 0.34    | 0.34   | GTPase activator activity | GO:005096 | 46             | 1.00E-04 | 0.0049         | GOBP slim         |
| 0.36                  | 0.36                   | 0.36    | 0.36   | GTPase activation | GO:005096 | 46             | 1.00E-04 | 0.0049         | GOBP slim         |
| 0.23                  | 0.23                   | 0.26    | 0.26   | Extracellular matrix | GO:0031012 | 28             | 1.50E-04 | 0.0115         | GOBP slim         |
| 0.13                  | 0.13                   | 0.14    | 0.14   | Ribosome        | GO:0003540 | 35             | 1.00E-04 | 0.0115         | GOBP slim         |
| 0.12                  | 0.12                   | 0.12    | 0.12   | Translation     | GO:0006412 | 44             | 2.00E-03 | 0.0226         | GOBP slim         |
| 0.10                  | 0.10                   | 0.10    | 0.10   | RNA polymerase II core promoter proximal region sequence-specific DNA binding | GO:0016801 | 44             | 3.40E-04 | 0.0157         | GOBP slim         |
| 0.15                  | 0.15                   | 0.15    | 0.15   | RNA polymerase II core promoter proximal region sequence-specific DNA binding | GO:0006678 | 44             | 3.40E-04 | 0.0157         | GOBP slim         |
| 0.18                  | 0.18                   | 0.18    | 0.18   | Proteinaceous extracellular matrix | GO:0003547 | 16             | 4.70E-03 | 0.0361         | GOBP slim         |
| 0.17                  | 0.17                   | 0.17    | 0.17   | SRP-dependent cotranslational targeting to membrane | REACT_953353 | 33             | 9.10E-05 | 0.0005         | GOBP (Reactome)   |
| 0.31                  | 0.31                   | 0.31    | 0.31   | L13a-mediated translation silencing of Ceruloplasmin expression | REACT_862151 | 31             | 3.60E-05 | 0.0008         | GOBP (Reactome)   |
| 0.26                  | 0.26                   | 0.26    | 0.26   | Zinc finger C2H2-type/integrin DNA-binding domain | IPRI01307 | 47             | 8.60E-07 | 0.0007         | GOBP (Reactome)   |
| 0.25                  | 0.25                   | 0.25    | 0.25   | Zinc finger C2H2 | PP00696     | 28             | 6.50E-05 | 0.0050         | GOBP (Reactome)   |
| 0.25                  | 0.25                   | 0.25    | 0.25   | Formation of a pool of free 40S subunits | REACT_100803 | 23             | 2.02E-04 | 0.0022         | GOBP (Reactome)   |
| 0.24                  | 0.24                   | 0.24    | 0.24   | RNA polymerase II core promoter proximal region sequence-specific DNA binding | GO:0010777 | 20             | 2.00E-04 | 0.0043         | GOBP (Reactome)   |
| 0.24                  | 0.24                   | 0.24    | 0.24   | RNA polymerase II core promoter proximal region sequence-specific DNA binding | GO:0010777 | 20             | 2.00E-04 | 0.0043         | GOBP (Reactome)   |
| 0.24                  | 0.24                   | 0.24    | 0.24   | Double-stranded DNA binding | GO:003690 | 30             | 1.00E-05 | 0.0056         | GOBP (Reactome)   |
| 0.24                  | 0.24                   | 0.24    | 0.24   | Double-stranded DNA binding | GO:003690 | 30             | 1.00E-05 | 0.0056         | GOBP (Reactome)   |
| 0.30                  | 0.30                   | 0.30    | 0.30   | Nononsense Mediated Decay (NMD) independent of the Exon Junction Complex | REACT_852436 | 16             | 2.20E-04 | 0.0023         | GOBP (Reactome)   |
| 0.30                  | 0.30                   | 0.30    | 0.30   | Nononsense Mediated Decay (NMD) enhanced by the Exon Junction Complex | REACT_124049 | 25             | 9.10E-06 | 0.0081         | GOBP (Reactome)   |
| 0.32                  | 0.32                   | 0.32    | 0.32   | Citrullination | GO:005191 | 20             | 1.50E-04 | 0.0402         | GOBP (Reactome)   |
| 0.36                  | 0.36                   | 0.36    | 0.36   | Response to a cAMP | GO:003540 | 9              | 8.30E-04 | 0.0344         | GOBP (Reactome)   |
| 0.36                  | 0.36                   | 0.36    | 0.36   | Response to a cAMP | GO:003540 | 9              | 8.30E-04 | 0.0344         | GOBP (Reactome)   |
| 0.38                  | 0.38                   | 0.38    | 0.38   | Trafic1osome Translation Termination | GO:0010475 | 13             | 3.60E-05 | 0.0065         | GOBP (Reactome)   |
| 0.38                  | 0.38                   | 0.38    | 0.38   | Trafic1osome Translation Termination | GO:0010475 | 13             | 3.60E-05 | 0.0065         | GOBP (Reactome)   |
| 0.40                  | 0.40                   | 0.40    | 0.40   | Basic-leucine zipper domain | GO:001700 | 11             | 2.10E-05 | 0.0167         | GOBP (Reactome)   |
Supplemental Experimental Procedures.
Bioinformatic Processing and Criteria.
Protein abundances and differential expression were evaluated using two approaches:
First, we compared protein abundances using a Label Free Quantitation (LFQ)-based
approach (parameter set in Supplementary Information). We did not use “Match-between-
runs” and increased the MQ-data point count to four data points. The data were processed
and statistically evaluated using the Perseus software package (ver. 1.5.2.6). Protein
localization for 5775 of 5940 newly-synthesized proteins were imported from the LocTree3
database (ver. 07-04-2016; Goldberg et al., 2014). Venn diagrams were generated using
VennDIS (Ignatchenko et al., 2015). After filtering for the presence in ≥ 2 biological
replicates (4471 proteins remaining) and for a coefficient of variation < 1 (4186 proteins
remaining), these proteins were annotated using UniprotKB (GOCC, GOMF, GOBP,
Reactome, Pfam, InterPro, protein families) and a 1D annotation enrichment was
performed (Perseus, Benjamini-Hochberg FDR = 0.05, both side test, Table S8; Cox et
al., 2012) and FunRich 2.12 (Benjamini-Hochberg FDR = 0.05 for enrichment analysis;
Pathan et al., 2016). Relative protein abundances analysis and statistical significance of
differential regulation between control and Bicuculline, control and Tetrodotoxin or
Bicuculline and Tetrodotoxin were calculated using an ANOVA test (S0=0.2, permutation-
based FDR = 0.05, 250 randomizations) yielding 307 differentially regulated proteins.
Proteins were mapped onto KEGG pathways (Kanehisa et al., 2016) using Incromap and
bioDBnet Converter, and pathway schemes were adapted and modified (http://www.genome.jp/kegg/).
Grey boxes in figures indicate detected proteins, asterisks show statistical significance in the Fisher LSD test (in Origin 2015G Sr1, OriginLab).
For visualization of the network between the 307 differentially regulated proteins, these
proteins were analysed using String (v. 10.0, string-db.org; Szklarczyk et al., 2015) with
experiments and databases as active interaction sources (textmining, co-expression,
neighborhood, gene fusion, co-occurrence disabled; minimum required interaction score:
medium, confidence 0.4, query proteins only). Proteins were plotted in custom pathway
schemes (modified from KEGG) and LFQ data depicted in call-out boxes for fold changes
> 0.5 (manual addition of selected candidates of interest). The 307 differentially regulated
proteins were analyzed for correlation with diseases using GeneAnalytics (“MalaCards”,
Lifemap Sciences), and association with diseases listed in brief in Figure 8 and in detail
in Table S7.
Second, we set out to analyze proteins identified exclusively in one condition. As no high-abundance proteins with high peptide counts and intensity values were detected exclusively, we analyzed proteins with low peptide and intensity counts that displayed a trend in abundance/detection for a specific condition. Here, we first manually evaluated candidates detected exclusively in 3/5 biological replicates in one condition and not detected in any other condition and examined their normalized peptide intensities to exclude detection-limit-based false positive hits. Only proteins displaying similar peptide counts and intensity profiles (based on normalized peptide intensity values) were retained for further analysis. All candidates including peptide counts, intensity values and fold-changes are listed in Table S2, page 2. We also made use of unique peptide ratios and normalized peptide intensity values for manually selected low-abundance proteins. We again focused on proteins with < 5 peptides in a group without robust LFQ data. We approximated abundance by comparing unique peptide counts and extracted proteins with $D > 0.6$ or $D < 0.4$ ($D = \frac{x}{x+y}$, with $x$ = peptides in condition A and $y$ peptides in condition B) and only retained proteins displaying similar trends in normalized intensity ratios. Due to the low peptide counts and intensity values available, the analyses described in the first part do not quantitative values, but rather suggest a trend in abundance.

**Supplemental references**

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