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Temporal Proteomic Analysis of Rat Hippocampus Provides Evidence for Memory Reconsolidation in Operant Conditioning

Análise Proteômica Temporal de Hipocampo de Ratos Fornece Evidências para a Reconsolidação de Memórias no Condicionamento Operante

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Table of Contents

Abbreviations

ACN – Acetonitrile
AcOH – Acetic acid
AMPA – α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANI – Anisomycin
CaMKII – Calcium/calmodulin-dependent protein kinase II
cAMP – Cyclic adenosine monophosphate
CREB1 – cAMP response element binding protein 1
DG – Dentate gyrus
DTT – Dithiothreitol
EC – Entorhinal cortex
E-LTP – Early-phase LTP
FC – Fear conditioning
FDR – False Discovery Rate
IAA – Iodoacetamide
iCAT – Isotope-coded affinity tags
ICR – Ion cyclotron resonance
IT – Ion trap
iTRAQ – Isobaric tags for relative and absolute quantification
LC – Liquid chromatography
LC-MS/MS – Liquid chromatography-tandem mass spectrometry
L-LTP – Late-phase LTP
LTP – Long term potentiation
MAPK – Mitogen activated protein kinase
MS – Mass spectrometry
m/z – Mass to charge ratio
NH_4AcO – Ammonium acetate
NH_4OH – Ammonium hydroxide
NMDA – N-methyl-D-aspartate
OC – Operant conditioning
OT – Orbitrap
PBS – Phosphate buffered saline
PCR – Polymerase chain reaction
PKA – Protein kinase A
PSD – Postsynaptic density
PTM – Post translational modification
SAX – Strong anionic exchange
SC – Subicular complex

SCX – Strong cationic exchange
SDS – Sodium dodecyl sulfate
SMT – Spatial memory tasks
TEAB – Triethylammonium bicarbonate
TFA – Trifluoroacetic acid
TMT – Tandem mass tags
TOF – Time-of-flight
UPS – Ubiquitin-proteasome system
XIC – Extracted ion chromatogram

Summary

Animals are able to store newly acquired information about the external world as memories. Memory formation occurs via rearrangements of neural circuitries, which are elicited by changes in transcription, translation and post-translation modifications (PTMs) in cells of specific regions of the central nervous system such as the hippocampus. Notably, the molecular characterization of memory formation has been carried out primarily in the context of animals that have been subjected to fear or spatial learning paradigms. In this study, we examined the molecular changes associated with information storage in rodents subjected to operant conditioning (OC). Herein, we employed strong anionic exchange (SAX) with salt gradient elution as a fractionation strategy followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) to measure changes in hippocampal proteome and phosphoproteome at early and late stages of memory formation, as well as after behavior recall. We identified a total of 8,951 proteins and 568 phosphoproteins, making this study the largest hippocampal proteome to date. Statistically significant abundance changes were shown in 465 proteins and 64 phosphoproteins throughout the aforementioned time intervals. Furthermore, quantitative polymerase chain reaction measurements of mRNA abundance levels revealed a weak interdependence between protein and transcript levels, giving credence to the notion of a low correlation between proteins and mRNAs in disturbed cellular states. Also, the identification of differentially regulated proteins of the ubiquitin-proteasome system (UPS), as well as calcium/calmodulin-dependent protein kinase II (CaMKII), provides evidence for the existence of a time window after behavioral recall in which stored information may become liable to further changes known as memory reconsolidation.

Summary in Portuguese

Os animais são capazes de guardar informações adquiridas do mundo exterior em forma de memórias. A formação de memórias ocorre através de um rearranjo de circuitos neurais, que é provocado por alterações na transcrição, tradução e adição de grupos químicos em proteínas em forma de mudanças pós-traducionais (PTMs) em células de regiões específicas do sistema nervoso central como o hipocampo. Notavelmente, a caracterização molecular da formação de memórias tem sido realizada primariamente em animais submetidos a paradigmas comportamentais relacionados a memória espacial ou de medo. Neste estudo, nós examinamos as mudanças moleculares associadas com o armazenamento de informações em animais submetidos ao condicionamento operante (OC). Aqui, empregamos a cromatografia de troca aniônica (SAX) *offline* com eluição através de um gradiente crescente de sal seguido de uma cromatografia líquida acoplada a espectrometria de massas em tandem (LC-MS/MS) para mensurar mudanças no proteoma e fosfoproteoma hipocampais em estágios precoce e tardio da formação de memórias, assim como depois da evocação do comportamento. Identificamos um total de 8.951 proteínas e 568 fosfoproteínas. Mudanças estatisticamente significativas foram detectadas em 456 proteínas e 53 fosfoproteínas ao longo dos intervalos de tempo mencionados anteriormente. Ademais, mensurações de abundância de mRNA por reação em cadeia de polimerase em tempo real revelou uma fraca interdependência entre os níveis de transcritos e proteínas, dando suporte a noção de uma baixa correlação entre proteínas e mRNAs em estados celulares perturbados. Além disso, a identificação de proteínas diferencialmente reguladas do sistema ubiquitina-proteassoma (UPS), assim como *calcium/calmodulin-dependent protein kinase II* (CaMKII), fornece evidência para a existência de uma janela de tempo depois da evocação do comportamento na qual informações armazenadas se tornam sensíveis a modificações conhecido como reconsolidação.

Extended summary of the work in Portuguese

O aprendizado e a memória são processos fundamentais na vida de um animal. O aprendizado refere-se à capacidade de adquirir novas informações sobre o mundo exterior, enquanto a memória é o mecanismo pelo qual esses dados são codificados e armazenados para uma evocação posterior. A formação de novas memórias depende do rearranjo de vias celulares específicas em diferentes regiões do sistema nervoso central como o hipocampo. Esses rearranjos incluem alterações morfológicas que são induzidas pela transcrição de genes específicos, a tradução de distintas sequências de mRNA e a regulação de proteínas por modificações pós-traducionais (PTMs). As moléculas e as regiões do hipocampo que medeiam essas mudanças podem diferir dado o tipo de informação que está sendo codificada. Por exemplo, animais submetidos a paradigmas comportamentais referentes a memória espacial sofrem modificações no giro denteado (DG), enquanto organismos sujeitos ao condicionamento contextual de medo (FC) possuem alterações nos neurônios da região CA1 do hipocampo. Entretanto, pouco se sabe sobre as mudanças referentes a formação de novas memórias no condicionamento operante (OC) – um paradigma comportamental no qual animais aprendem a associar um comportamento com a sua consequência (e.g. roedores pressionando uma alavanca para liberar água ou comida em uma caixa de Skinner).

Esse processo de formação de memórias pode levar dias. Durante esse tempo, memórias instáveis se tornam progressivamente estáveis ao longo do tempo por um processo conhecido como consolidação. Estudos tem mostrado que o potencial de longa duração (LTP), que pode ser definido como um fortalecimento da conexão sináptica entre neurônios, é o principal mecanismo celular do processo de consolidação em FC e paradigmas comportamentais referentes a memória espacial. O potencial de longa duração tem sido dividido em dois estágios: precoce e tardio. Em um modelo comumente utilizado, a fase precoce do LTP (E-LTP) é guiada primariamente por eventos de transdução de sinais. Durante as primeiras horas que seguem a aquisição de uma memória, íons de Ca^{2+} entram no neurônio pós-sináptico e ativam diversas cinases como a *calcium/calmodulin-dependent protein kinase II* (CaMKII). CaMKII ativada fosforila resíduos de receptores localizados na membrana pós-sináptica, aumentando a condutância deles. Essa ativação da CaMKII também leva ao tráfico de receptores do tipo *α-amino-3-hydroxy-5-methyl-*

4-isoxazolepropionic acid (AMPA) para as densidades pós-sinápticas (PSDs), uma estrutura especializada localizada na membrana dos neurônios pós-sinápticos. De 3 a 6 h depois da aquisição da memória, a fase tardia do LTP (L-LTP) inicia-se, desencadeando síntese proteica e grandes alterações do proteoma do hipocampo. Por exemplo, a *protein kinase A* (PKA) ativa fatores de transcrição incluindo *cyclic AMP-responsive element-binding protein* (CREB). CREB ativado aumenta a transcrição de genes alvo que medeiam mudanças estruturais nos neurônios pós-sinápticos.

Interessantemente, estudos iniciais mostraram que a administração de inibidores de síntese proteica como *anisomycin* (ANI) em camundongos antes ou imediatamente após serem submetidos a paradigmas comportamentais resultaram em um déficit na formação de memórias. Entretanto, administração de ANI 1 h depois do termino da sessão de treinamento não afetou a consolidação de memórias. Esses resultados indicam que deve existir uma janela prematura para a síntese de proteínas – isso é, a síntese *de novo* de proteínas durante os estágios iniciais do E-LTP é necessário para a consolidação de memórias. Ademais, memórias consolidadas ainda podem sofrer mudanças quando o comportamento é evocado novamente. Esse processo, conhecido como reconsolidação da memória, induz a tradução de novas proteínas que causam modificações adicionais na arquitetura dos neurônios do hipocampo. Acredita-se que o processo de reconsolidação da memória é uma adaptação dos organismos para fornecer novas informações ao conhecimento que já foi adquirido anteriormente, aumentando as chances dos animais de sobreviver em um mundo em constante mudança. O processo de reconsolidação da memória foi demonstrado em uma série de tarefas comportamentais, mas evidências desse processo em ratos submetidos ao OC ainda estão em discussão.

Com o intuito de entender o processo de consolidação de memórias em ratos submetidos ao OC, empregamos *in-depth* cromatografia líquida acoplada a espectrometria de massas em tandem (LC-MS/MS) para medir mudanças em proteínas e fosfoproteínas hipocampais a 30 min e 12 h depois de submeter animais ao condicionamento operante. Ademais, sondamos o proteoma e fosfoproteoma desses animais depois da evocação desse comportamento um dia depois. Identificamos um total de 8.951 proteínas e 568 fosfoproteínas, das quais 465 e 64 mostraram mudanças estatisticamente significativas, respectivamente. Aqui, revelamos a

identidade de proteínas que exibiram variações significativas tão cedo quanto 1 h depois do treinamento e demonstramos que as variações de abundância nas proteínas não foram acompanhadas por uma mudança nos níveis de seus transcritos. Ademais, fornecemos evidências moleculares para a existência de mecanismos de reconsolidação, indicando que o armazenamento de um comportamento OC provavelmente também sofre modificações a fim de atualizar memórias adquiridas previamente.

Introduction

a. The Molecular Bases of Memory

Memory is one of the most important components of behavior and it refers to the capacity to store and retrieve new information about the physical world (Kandel et al., 2014). Memory has emerged very early in the evolutionary history of the nervous system, and it has played a central role in helping organisms to adapt and survive the challenges faced in their environments (Emes et al., 2008; T. J. Ryan and Grant, 2009). This cognitive process has reached one of its most complex forms in humans, where deficits can have devastating consequences for the individual. In Alzheimer disease, for instance, the buildup of misfolded proteins in the brain disrupts the connectivity between nerve cells, leading to memory impairments and dementia (Musunuri et al., 2014; C. A. Ross and Poirier, 2004). In Huntington's disease, likewise, the expansion of a CAG triplet in the huntingtin gene leads to motor abnormalities and cognitive decline such as memory loss (Pontes and de Sousa, 2016). As a result of that, memory has been the focus of intensive investigation in different research fields such as psychology, anthropology and neuroscience (Rempel-Clower et al., 1996; Tronson and Taylor, 2007; Zola-Morgan et al., 1986).

The first scientific studies on memory were performed in patients with lesions in specific brain regions. One of the most famous cases is of Henry Molaison, also known as patient H.M. Suffering from constant epileptic episodes, Molaison had two thirds of his medial temporal lobes removed in an attempt to control his seizures. Although the surgery was a success, H.M. was incapable of forming new memories (Moser et al., 2015). Moreover, model organisms were also used in these initial studies. Typically, these animals were subjected to behavioral paradigms such as fear conditioning (FC) – a form of associative learning in which organisms pair an aversive stimulus such as a foot shock with a neutral context such as a box or a sound – followed by administration of drugs such as protein synthesis inhibitors or lesions in specific regions of the central nervous system in an attempt to identify brain areas related to memory (Strekalova et al., 2003). Notably, these assays revealed that the hippocampus possessed an important role in the storage of newly acquired information (Pontes and de Sousa, 2016).

The hippocampus is a structure located bilaterally in the medial temporal lobe of the vertebrate brain (Bliss and Collingridge, 1993). In rodents, it can be anatomically divided into a few distinct regions: the dentate gyrus (DG), the entorhinal cortex (EC), the subicular complex (SC) and the hippocampus proper, which is composed of the CA1, CA2, CA3 and CA4 areas (Figure 1; Strange et al., 2014). Each of these sections harbors different populations of cells that communicate through two main pathways, namely the direct perforant pathway and the trisynaptic circuit, also known as the indirect perforant pathway (Pontes and de Sousa, 2016). The former transmits multimodal sensory and spatial information directly from the EC to the CA1 area, a major output of the hippocampus. By contrast, in the trisynaptic circuit, information is sent from the EC to the CA1 area following this route: EC→ DG → CA3 → CA1 (Figure 1; Neves et al., 2008; Pontes and de Sousa, 2016). Interestingly, the submission of animals to different types of behavioral paradigms elicits morphological changes in distinct regions of the hippocampus. For instance, spatial memory tasks (SMTs) generally induce changes in the DG, whereas FC elicits alterations in the CA1 neurons (Monopoli et al., 2011; Strekalova et al., 2003).

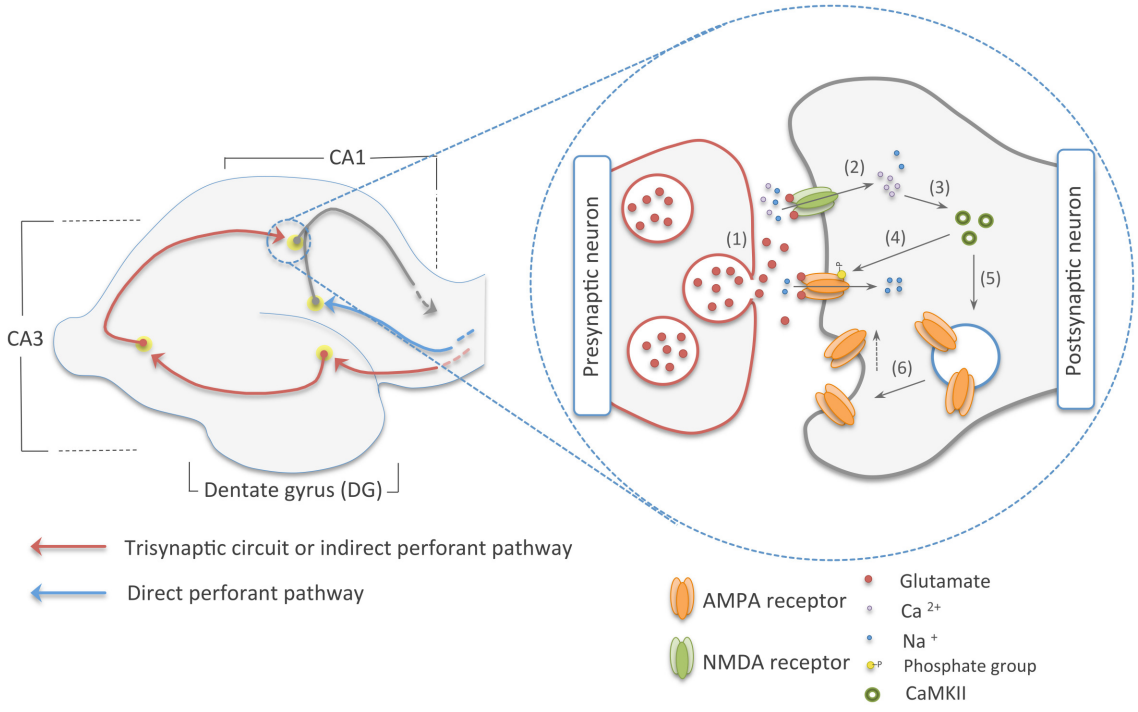

FIGURE 1 I The two main pathways to the CA1 area of the hippocampus on the left, and early-phase NMDA dependent-LTP on the right. The red arrows in the picture on the left show the trisynaptic circuit of the hippocampus, where multimodal sensory and spatial information coming from the entorhinal cortex (EC) is relayed to the CA1 area following this route: EC–DG–CA3–CA1. In blue, we illustrated the

direct perforant pathway, which directly connects the EC to the CA1 region. On the picture in right, we show an illustration of the early-phase LTP. Here, (1) glutamate from the presynaptic neuron is released into the synaptic cleft. (2) This neurotransmitter reaches ionic channels of the postsynaptic cell causing depolarization of this neuron by the influx on sodium and calcium cations. (3) Calcium, in its turn, activates CaMKII that (4) phosphorylates ionic channels in the PSDs and (5, 6) induces the addition of AMPA receptors to the postsynaptic membrane, increasing synaptic efficiency.

The morphological changes associated with the formation of new memories in the hippocampus are triggered by the transcription of specific genes, the translation of distinct mRNA sequences, and the regulation of proteins by post-translational modifications (PTMs) (Kaltschmidt et al., 2006; Ma et al., 2014; Park et al., 2006). Notably, the formation of a new memory may take up to several days. During this time, liable memories become stable over time through a process known as memory consolidation (Squire et al., 2015). Studies have shown that long-term potentiation (LTP), which can be defined as an enhancement of synaptic strength, is the major cellular mechanism underlying the process of memory consolidation in FC and SMTs (Nabavi et al., 2014; Uzakov et al., 2005; Whitlock et al., 2006). LTP has been generally divided into two distinct stages: an early phase that is driven primarily by signal transduction events and a late phase which triggers *de novo* protein synthesis (Abel et al., 1997a; Granger et al., 2013).

Early-phase LTP (E-LTP), also referred as the induction phase, begins by the release of glutamate from the presynaptic terminal into the synaptic cleft. Glutamate, the main excitatory neurotransmitter in the brain, diffuses through the cleft and reaches the postsynaptic neuron, where it binds to ligand-gated ion channels. The interaction between the neurotransmitter and the ionotropic N-methyl-D-aspartate (NMDA) receptor results in the influx of Ca^{2+} and Na^{+} into the cell (Castillo, 2012; Dingledine et al., 1999; Schiller et al., 1998). The Na^{+} helps to bring about a depolarization of the postsynaptic neuron that last a few milliseconds, while the Ca^{2+} promotes the activation of protein kinases such as calcium/ calmodulin-dependent protein kinase (CaMKII) (Lisman et al., 2012; Lüscher and Malenka, 2012). CaMKII and other kinases prompt the introduction of other ionotropic channels such as α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors into the postsynaptic density (PSD) – a specialized membrane bound structure located in postsynaptic neurons (X. Chen et al., 2008; Hayashi et al., 2000; W. Lu et al., 2010). This traffic of new AMPA receptors to the membrane ultimately leads to an improvement in synaptic communication (Figure 1).

If the activation of the pre and postsynaptic neurons persists for longer periods of time – something that can also be accomplished *in vitro* by repeated stimulation of the cells by high frequency tetanus pulses of 100 Hz –, a number of signaling cascades are activated, leading to *de novo* protein synthesis (Hölscher et al., 1997; Nguyen et al., 1994; T. J. Ryan et al., 2015). This is known as late-phase LTP (L-LTP), also called the expression phase. In this stage, the rise in Ca^{2+} ions inside the cell, caused by the constant release of glutamate by the presynaptic cell, induces the increase in the production of cyclic adenosine monophosphate (cAMP) by adenylyl cyclase (Poser and Storm, 2001; Wong et al., 1999). cAMP, in turn, activates protein kinase A (PKA) that switches on mitogen activated protein kinase (MAPK) (Abel et al., 1997a; Roberson et al., 1999). This kinase is translocated to the nucleus and phosphorylates cAMP response element binding protein 1 (CREB-1), an important transcription factor (Patterson et al., 2001; Viola et al., 2000). The phosphorylation activates CREB-1, resulting in increased transcription of a number of target genes and their subsequent translation into proteins responsible for the formation of new synaptic connections (Figure 2; Ahmed and J. U. Frey, 2005a; Deisseroth et al., 1996; Pontes and de Sousa, 2016).

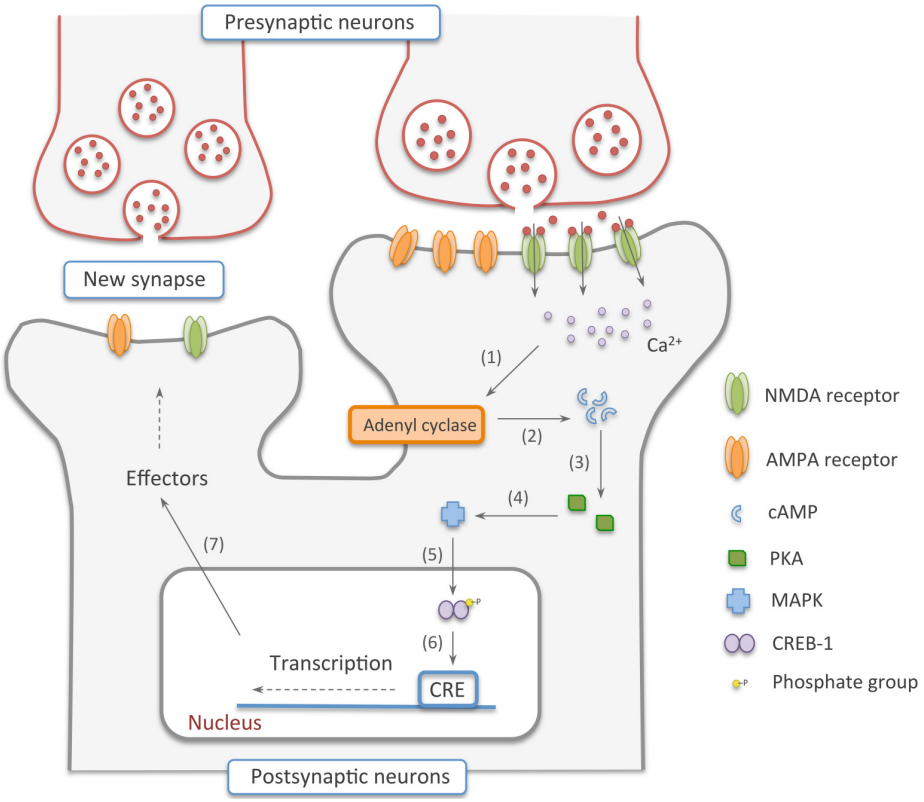

FIGURE 2 I Late-phase LTP. In this stage, (1, 2) Ca^{2+} ions inside the cell recruit adenyl cyclase to produce cAMP. (3, 4) Cyclic adenosine monophosphate, in turn, activates PKA that switches on MAPK. (5) This kinase is translocated to the nucleus and phosphorylates CREB-1, an important transcription factor. (6, 7) The phosphorylation activates CREB-1, resulting in increased transcription of a number of target genes and their subsequent translation into proteins responsible for the formation of new synaptic connections.

Interestingly, however, consolidated memories can still undergo changes or even disruption upon recall in a process known as memory reconsolidation (J. L. C. Lee et al., 2017). During reconsolidation, the retrieval of a specific memory can induce the translational of new proteins and the degradation of pre-existing ones (S.-H. Lee et al., 2008; Tronson and Taylor, 2007). These molecular events lead to extra modifications in the architecture of neurons in the hippocampus. Memory reconsolidation is believed to be an adaptation of organisms to supply additional information to knowledge that has been previously acquired, increasing the chances of animals to survive an everlasting changing world (J. L. C. Lee et al., 2017). This process has been demonstrated in a plethora of behavioral tasks, but evidence for it in animals subjected to operant conditioning (OC) – a type of behavioral paradigm wherein animals learn to associate a behavior with its consequence (e.g. rodents pressing a lever for the release of water or a food pellet in a Skinner box) – is still under debate (Hernandez and Kelley, 2004; Tronson and Taylor, 2007).

b. Mass Spectrometry (MS)-based Proteomics

Proteomics is a system-wide analysis of the proteins expressed in a specific cell, tissue or organism at a given time (N. G. Anderson and N. L. Anderson, 1996; Zhang et al., 2013). Although the term relates to any technology that seeks to interrogate a large number of proteins, today proteomics is used to name works where the central platform is mass spectrometry (MS). Currently, the gold standard strategy in MS-based proteomics is shotgun proteomics (Domon and Aebersold, 2010; Mann and Kelleher, 2008). Here, a complex mixture of proteins is digested into peptides with a protease of interest – usually trypsin, which cleaves on the C-terminal of lysine and arginine. Subsequently, the peptides are separated online by reverse-phase liquid chromatography (LC) and analyzed by high resolution mass spectrometers such as time-of-flight

(TOF), orbitrap (OT) or ion cyclotron resonance (ICR)(Beck et al., 2015; Marshall et al., 1998; Michalski et al., 2012; Thakur et al., 2011).

In a typical shotgun experiment, the LC-MS/MS run takes up to 120 min and is composed of thousands of cycles, each made of a MS1 scan – also known as a full scan – that measures the peptides' mass to charge ratio (m/z) and intensity, and a MS2 or MS/MS scan. During the MS2 scan, the 20 most intense peptides in each cycle are fragmented in a collision cell, usually filled with an inert gas such as nitrogen or helium, and their spectra are again measured to obtain sequence information (Geiger et al., 2011; Thakur et al., 2011). Once acquired, the MS data is used in searches within databases containing peptides digested *in silico* to identify the proteins present in the sample(s) (Figure 3; Sadygov et al., 2004).

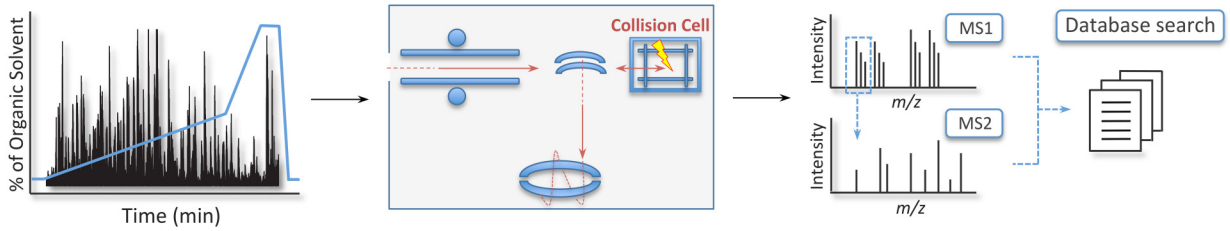

FIGURE 3 I Workflow of the gold standard strategy in shotgun proteomics. Here, peptides are separated online in a reverse-phase liquid chromatography and electrosprayed into the mass analyzer. The mass spectrometer measures the peptides' m/z and intensity in the MS1 cycle. Upon fragmentation, the product ions of each peptide are reanalyzed to obtain sequence information of the analyte in the MS2 cycle. Once this experimental data is acquired, information is searched against a database of the organism of interest to identify the proteins in the sample.

In addition to protein identification, mass spectrometry can be used to extract quantitative information from samples. Protein quantification can be absolute – if known amounts of a heavy analog of the analyte of interest is added prior to the analysis in a mass spectrometer – or relative, if samples in different states are compared (e.g. brain tissue from rodents trained in a behavioral paradigm and controls) (Bantscheff et al., 2012; Kettenbach et al., 2011). The most popular strategies for relative quantification are label-free, metabolic labeling, and chemical labeling (Figure 4).

In label-free quantification, as the name suggests, no label is added to the samples, which are digested and run separately in the mass spectrometer – being the results combined after the acquisition of the data (Filiou et al., 2012). In this strategy, quantification takes advantage of the

area plotted over time for each ion as it elutes from the chromatographic column. Later, this extracted ion chromatogram (XIC) is aligned across different samples and a ratio for each peptide is obtained. Another mode of quantification in label-free experiments is spectrum counting. Here, quantification is based on the number a particular peptide is fragmented during a LC-MS/MS run, which serves as a proxy for abundance and can be compared between conditions(Bantscheff et al., 2007). Label-free is the least accurate strategy of relative quantification, but it has gained momentum due to its low cost, improvements in sample handling, refinement of the chromatographic setup, and development of software for data analysis (Altelaar and Heck, 2012; Ong and Mann, 2005).

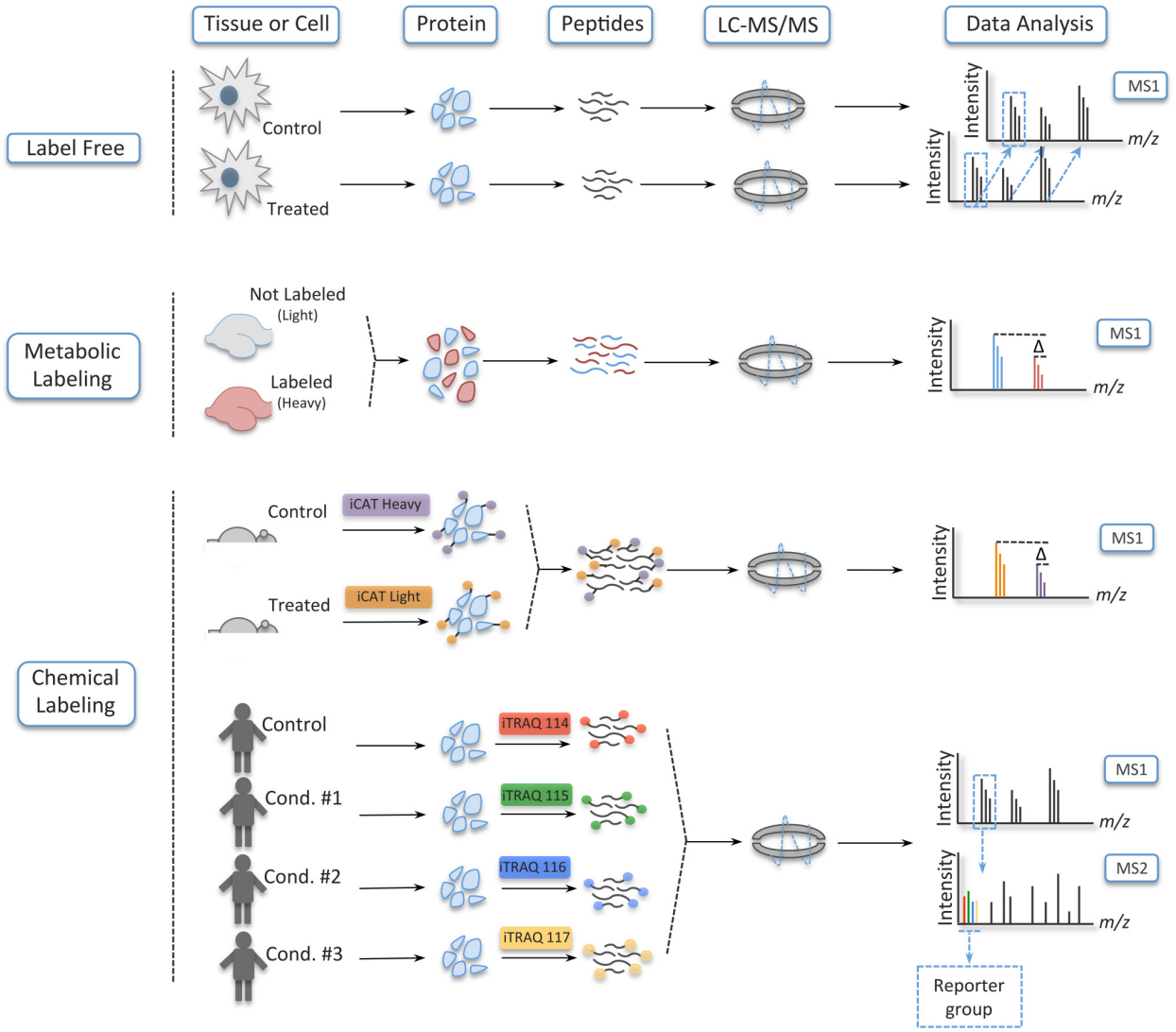

FIGURE 4 I Different strategies to quantify peptides. In label-free experiments, samples are digested and ran separately in a mass spectrometer; they are combined only in the data analysis. In metabolic labeling, on its turn, one of the conditions is grown in medium containing amino acids labeled with heavy isotopes or heavy nitrogen. Here, the samples are combined very early, and sample handling and analysis are done concomitantly. Lastly, in chemical labeling, tags are incorporated at the protein level, as is the case of iCAT, or at the peptide level with iTRAQ and TMT. In these strategies, the samples are combined early in the workflow of the experiment without increasing the complexity of the samples in the MS1, since the tags of iTRAQ and TMT are isobaric—being distinguished only upon fragmentation in a collision cell.

By contrast, in metabolic labeling, prior to protein extraction at least one of the conditions is labeled with a heavy stable isotope such as ^{15}N or heavy amino acids such as lysine, arginine or both (Ong et al., 2002; Rauniyar et al., 2013). The use of a heavy analog prevents the variation usually encountered in label-free experiments, since the samples are mixed, digested and analyzed simultaneously in the LC-MS/MS run. This can be accomplished because heavy and light, non-labeled, peptides retain the same physicochemical properties (e.g. retention time during the LC), but a mass shift between them enables their distinction latter in the data analysis. The only exception to this rule is deuterium (^{2}H), which is more hydrophilic than hydrogen; this creates a delay in the retention time between the labeled and non-labeled conditions (Yi et al., 2005). Here, as is also the case in label-free experiments, quantification is acquired by the peak area ratios of the heavy and light peptides in the XIC (Bantscheff et al., 2007; Ong et al., 2002).

Even though metabolic labeling is the most accurate relative quantification strategy, it has a restricted capacity to multiplexing due to the limitation on the isotopes that can be added to an amino acid and the increase in sample complexity in the MS1 (Hebert et al., 2013). Chemical labeling, in its turn, is able to circumvent those limitations. To this day, many chemical labeling reagents have been developed, but the most used are isotope-coded affinity tags (iCAT), tandem mass tags (TMT) and isotope tags for relative and absolute quantification (iTRAQ) (Gygi et al., 1999; P. L. Ross et al., 2004; Thompson et al., 2003). iCAT labels samples at the protein level, and the tags are composed of a reactive group that binds to reduced cysteine residues, a linker group that incorporates isotopes in the heavy reagent, and a biotin affinity group for the isolation of the iCAT-labeled peptides. In a typical experiment using iCAT, two conditions are labeled with the light and heavy version of the tags, mixed, and enzymatically cleaved. Next, the peptides with the tags are enriched by avidin affinity chromatography and analyzed in a LC-MS/MS run. Here, quantification is obtained by the peak area ratios of the heavy and light peptides (Yi et al., 2005).

iCAT possesses the same limitation in multiplexing as metabolic labeling, yet iTRAQ and TMT enables from 8 to 10 samples, respectively, to be analyzed in a single experiment. iTRAQ and TMT are isobaric tags and label analytes and the peptide level. Their tags are composed of a reactive group that binds to the N-terminal of peptides and lysine residues, a balance group – which ensures that the same peptides in the different conditions elute together and are indistinguishable in the MS1 scan –, and a reporter group. Unlike other quantitative strategies, quantification on iTRAQ and TMT is based on the intensity signal of the reporter group that is released from the analytes upon fragmentation in a collision cell. Nevertheless, chemical labeling also has limitations and some considerations have to be taken to get around these drawbacks (For in depth information on iTRAQ and TMT strategies see Bantscheff et al., 2008; Karp et al., 2010; Ting et al., 2011; Wenger et al., 2011).

The shotgun quantitative strategies described above have fostered a revolution in many fields of biology such as cancer, immunology, and neuroscience by improving our understanding of the systemic cellular response of stimulated or disease states vs. control (Boersema et al., 2013; Geiger et al., 2012; Kruger et al., 2008; Meissner and Mann, 2014). Yet, when it comes to the proteomic study of memory, very few MS-based experiments have been carried out so far (Borovok et al., 2016; Monopoli et al., 2011). Some authors believe that such discrepancy is due to the difficulty to characterize proteins that are genuinely associated with this cognitive process due to high biological variability among individuals within areas related to memory such as the hippocampus. Nevertheless, this explanation falls short, since transcriptomics assays to investigate memory have been conducted before with success (Cavallaro et al., 2002). Moreover, a few proteomic experiments have been carried out in animals subjected to FC and SMTs, yet none in animals subjected to operant conditioning.

Motivations for This Work

Memory is one of the most important components of cognition, yet It has been largely studied in animals subjected to specific behavioral paradigms such fear conditioning (FC) or spatial memory tasks (SMTs). Moreover, most of these assays have employed techniques that are limited to a specific sequence such as gene knockouts or administration of protein inhibitors, lacking understanding of the process of memory consolidation at a global-scale. Transcriptomic assays have been performed as well, however transcripts are not the end products of most biological functions and can only be used as a proxy for protein dynamics.

In order to extend the knowledge of how memories become consolidated over time in the hippocampus of animals subjected to an operant conditioning (OC) task, we measured protein, phosphoproteins and mRNA levels in rats subjected to this behavioral paradigm at different time points. To accomplished that, we performed the following steps throughout this studied:

- Modify the standard behavioral protocol of OC to identify proteins, phosphoproteins and transcripts which are genuinely associated with this form of memory;
- Develop and implement a pre-fractionation strategy for in-depth identification of the proteins in the hippocampus via LC-MS/MS;
- Identify statistically significant abundance changes at 30 min, 12 h and after a recall session the took place 24 h following behavioral conditioning;
- Select mRNA targets based on these results and the literature to understand the relation between protein and transcript levels;
- Identify and quantify the phosphoproteome of rats at the aforementioned time points using mass spectrometry.

Results

Parameters of the Operant Conditioning Paradigm

In order to measure quantitative changes in protein, phosphoprotein and mRNA during memory consolidation and after recall, we randomly distributed rats (n = 12) into four different groups. All of these animals, except the control individuals, were subjected to OC in a Skinner box, with a minor modification of the standard protocol (Figure 5A). In a commonly used training schedule, rodents are placed in the box for two or three 20 min sessions (i.e. habituation) prior to conditioning. This is done to minimize stress responses that can negatively affect the training procedure, since some animals, for instance, may freeze when placed into a new environment. During habituation, a reward such as a food pellet is released at random by a feeder. After that, habituated animals return to the box and undergo training trials, which consist of exposing a retracted lever every 20 s for 60 s and measuring the number of correct lever presses of each animal (Exton-McGuinness et al., 2014; Rapanelli et al., 2011).

In this standard procedure, however, the criterion for behavior acquisition is not usually reached in a single training day, such that the animals have to be subjected to additional trials in the Skinner box. In some cases, conditioning sessions may take up to 10 days (Exton-McGuinness et al., 2014; Jurado-Parras et al., 2013). We anticipated this to be problematic because subjecting rodents to consecutive training sessions could potentially dilute proteomic and transcriptomic signals associated with the process of memory consolidation or molecular signatures correlated with memory reconsolidation mechanisms. We thus manually performed the training by releasing water (i.e. the reward) as animals got progressively closer to the lever. Eventually, animals learnt that pressing the lever in the box would result in the reward (Figure 5B). Here, we set the criterion for behavioral acquisition to 15 lever presses in a row (Figure 5C). Animals subjected to this modified training protocol were able to retain the information of a single training day for a couple of days, while individuals that were subjected to a recall session (i.e. 50 lever presses in a row) remembered the event for at least two weeks (Figure 5C; data not shown). These results show that rats are able to learn OC tasks in one training session, establishing the use of this modified protocol to study associative memories in the hippocampus.

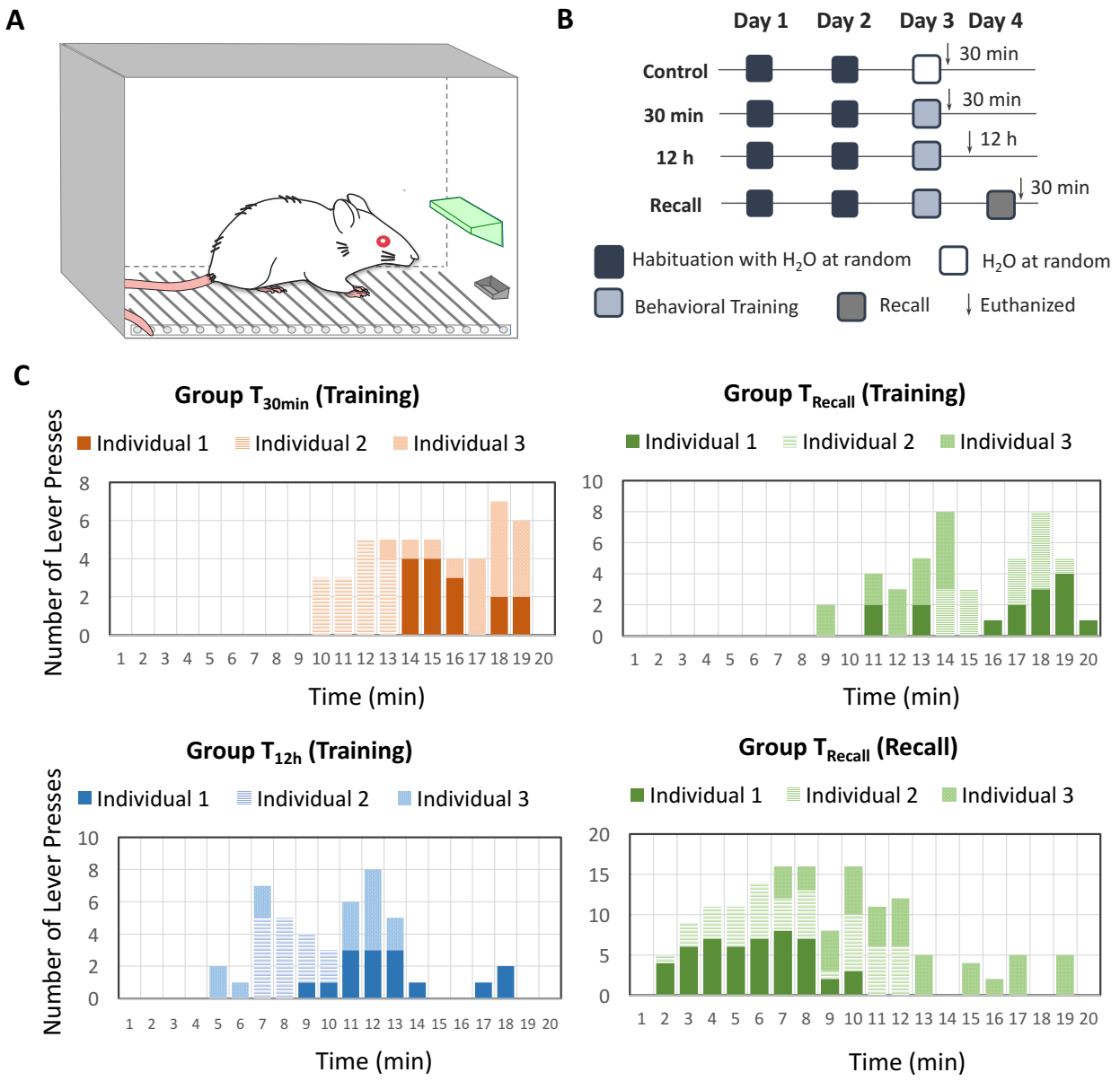

FIGURE 5 I Parameters of the Operant Conditioning Paradigm. (A) Illustration of a Skinner box. (B) Behavioral protocol used in the different groups of the experiment. (C) Number of lever presses of each animal in the different groups – 30 min, 12 h and Recall – during a 20 min session. Animals pressed 15 times the lever during the training session, while rodents subjected to a recall session pressed the lever 50 times. Control individuals were not subjected to training, but underwent habituation just like the other animals of the experiment.

Strong Anion Exchange (SAX) Fractionation Enables In-depth Proteomics of the Hippocampus

Rats subjected to the aforementioned training protocol were euthanized at distinct time points – 30 min, 12 h and 30 min after a recall session performed one day after conditioning. Control animals did not undergo OC, but were placed in the Skinner box like the other animals.

These control rats also went through the habituation steps described earlier, being euthanized 30 min after leaving the box (Figure 5B). The hippocampi of all animals were dissected under 3 min and processed using the filter-aided sample preparation (FASP) method, with minor modifications (Figure 6A; see methods for more details). After tryptic digestion, peptides of each condition were labeled with isobaric tags for relative and absolute quantification (iTRAQ) in biological triplicates – control (tag 114), 30 min (tag 115), 12 h (tag 116), recall (tag 117). Next, labeled peptides derived from each condition were mixed in equal amounts, and small aliquots from each batch were analyzed in short LC-MS/MS runs. This was performed to verify potential differences in the efficiency of the labeling reactions and to measure the proportion among the iTRAQ tags in each batch. As illustrated in Figure 6B, the labeling was carried out effectively and the proportion among the tags have been evenly distributed after normalization.

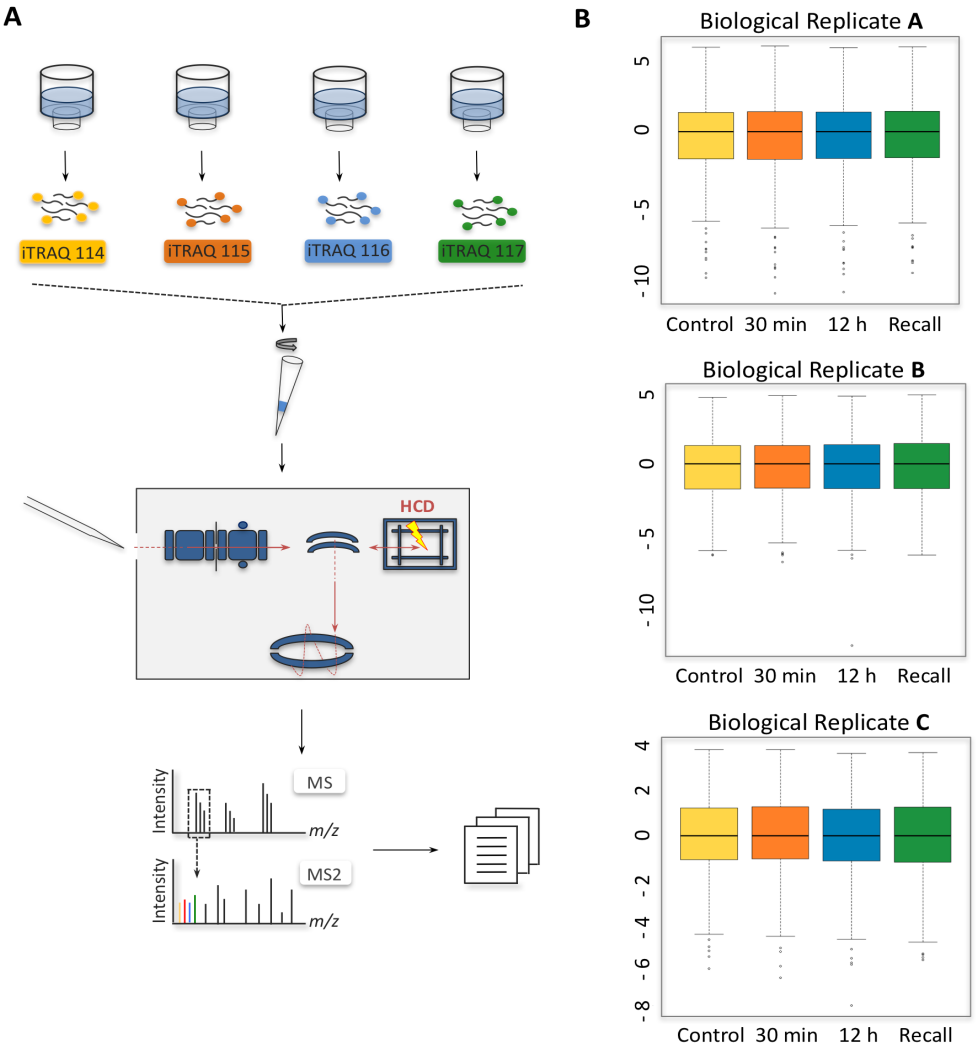

FIGURE 6 I Strong Anion Exchange (SAX) Enables In-depth Proteomics of the Hippocampus. (A) Workflow of the experiment. Hippocampi samples from operant conditioned and control animals were enzymatically digested, labelled with different iTRAQ reagent tags and combined in equal ratios before SAX fractionation. Fractionated samples were run separately in the mass spectrometer and the data were combined computationally. (B) Prior to fractionation, each batch of the biological replicates containing labelled peptides were analyzed in short LC-MS/MS to verify labeling efficiency and proportion among the tags. After normalization, the proportion among the tags have been distributed evenly in all replicates.

Since animal proteomes exhibit great complexity and large dynamic ranges (Mann et al., 2013; H. Wang et al., 2015), we decided to pre-fractionate samples prior to mass spectrometry analyses. Before performing this step in the iTRAQ labelled samples, we benchmarked for the first time two offline fractionation strategies: strong anion exchange (SAX) and strong cation exchange (SCX). Differently from previous assays that have used the isoelectric point (pI) to separate peptides in SAX (Nagaraj et al., 2011; Wisniewski et al., 2009), we eluted the analytes from the stationary phase by increasing the concentration of salt in the elution buffers. Label-free analyses of the fractions showed that SAX fractionation was superior to SCX, although the later has been the method of choice in most proteomic experiments to date (Azimifar et al., 2014; Villén et al., 2007). The SAX approach yielded a much larger number of identifiable proteins and also resulted in highly reproducible technical replicates when compared with the SCX strategy (average Pearson correlation coefficient of 0.9; Figure 7; supplementary excel file 1). Thus, we used SAX to separate the iTRAQ labeled samples into 12 fractions per biological replicate.

Subsequently, each SAX fraction was analyzed using a 165-min organic gradient in a hybrid-ion trap orbitrap mass analyzer (Orbitrap Elite). All the raw files were processed in Proteome Discover (PD) version 2.1 (Thermo Fisher Scientific) using Sequest HT and AmandaMS as searching engines, setting the false discovery rate (FDR) at the protein and peptide levels at 1%. Using these search parameters, we identified a total of 7,744 proteins with at least one unique peptide and an average sequence coverage of 17.5% (supplementary excel file 2). The data generated through the benchmarking of SAX and SCX in combination with the results of the iTRAQ labeled samples produced a total of 8,951 proteins. This data set shows a 13% increase of coverage in comparison to a previously mouse hippocampal proteome study, making the present work the largest hippocampal proteome data set to date (Sharma et al., 2015).

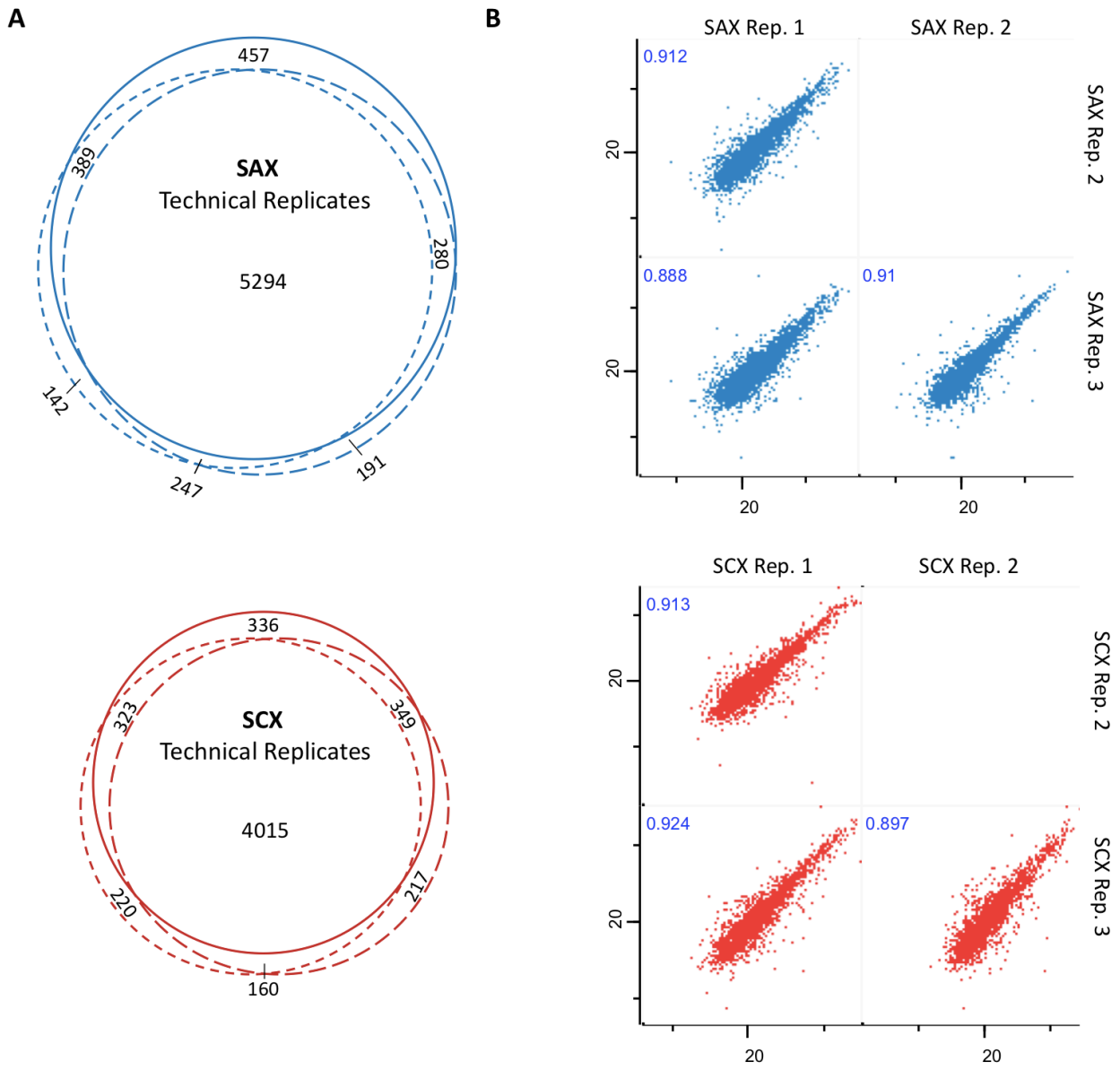

FIGURE 7 I Benchmark of SAX to SCX. Hippocampal peptides coming from the same digestion batch were divided equally into six microtubes – SCX (n = 3) and SAX (n = 3). They were dried in a Speedvac and re-suspended with the appropriate buffer (see methods for more details). These peptides were loaded into the columns and eluted by increasing the concentration of salt in the mobile phase. Next, each fraction was desalted utilizing StageTips containing C_{18} disks and analyzed separately in the mass spectrometer. (A) Venn diagrams of the technical replicates of SAX and SCX strategies. In total, we identified 7,000 proteins using SAX and 5,620 proteins using SCX. (B) Density plots of the technical replicates of the two fractionation strategies; Pearson correlation is shown in blue in each quadrant. Average Pearson correlation for SAX was 0.9 and for SCX was 0.91. We repeated these experiments with different elution buffer concentration and distinct organisms (i.e. *Trypanosoma cruzi* and HeLa cells) (data not shown). We obtained similar results in all experiments.

Quantitative Proteomics of the Hippocampus During Memory Formation

Quantitative information about proteins identified at the three-time intervals was obtained by extracting iTRAQ intensities derived from high confidence hippocampus peptides. Evaluation of these intensities in the R software enabled the quantification of 4,554 proteins, which spanned the fold-change range of $-2 \leqslant X \geqslant 2$ (supplementary excel file 3). Proteins displaying significant abundance changes were identified combining limma test and rank products. These statistical tools work well in experiments with a small number of biological replicates (n = 3) and data sets with additional missing values such as transcriptomic and proteomic assays (Schwämmle et al., 2013). These analyses yielded a total of 465 regulated proteins throughout the three-time points under study here ($p < 0.05$; supplementary excel file 3).

To find similarities across the experimental conditions and to uncover putative functional relationships among all the quantifiable proteins, we subjected them to fuzzy c-means clustering. By determining the fuzzifier according to (Schwämmle and Jensen, 2010), we compiled the data set into three clusters (Figure 8; supplementary excel file 4). The proteins in cluster number 1 exhibited an increase in abundance throughout the process of memory consolidation and after the recall session, while proteins in cluster number 2 showed an opposite tendency. Conversely, proteins in cluster number 3 decreased 30 min after conditioning, but increased once again at 12 h and after behavior retrieval.

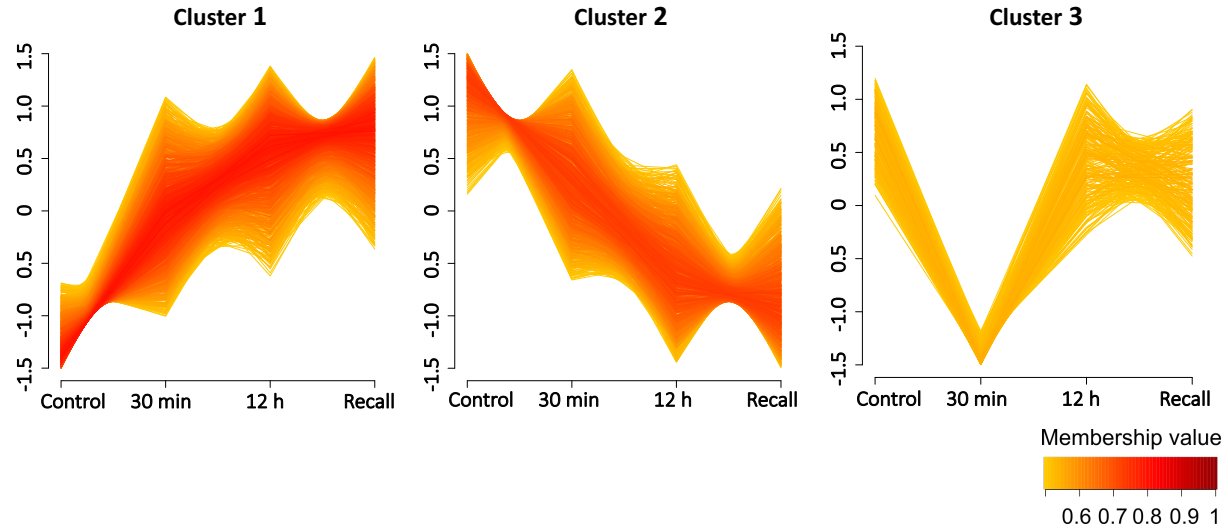

FIGURE 8 I Fuzzy c-means Clustering. Determination of the fuzzier enabled grouping of proteins into three distinct clusters, which show patterns of expression across time. The proteins in cluster 1 display an increase in abundance throughout the process of memory consolidation and after the recall session. Proteins of cluster 2 exhibited an opposite trend to cluster 1. Proteins of cluster 3 decreased their abundance levels 30 min after conditioning, but increased once again at 12 h and after behavior retrieval.

Proteins Displaying Abundance Changes as Early as 30 min After Operant Conditioning

Administration of protein synthesis inhibitors prior to, or just after subjecting animals to behavioral paradigms, leads to deficits in memory consolidation (Abel et al., 1997b; Bourtchouladze et al., 1998). This demonstrates that *de novo* protein synthesis is required during such a time interval for the establishment of long-term memory. However, because translation inhibitors possess a broad-spectrum, the identity of many protein(s) necessary at early stages of memory consolidation remains unknown. To probe the identity of these molecular players, we analyzed the proteomic data set at 30 min after conditioning and showed that 20 proteins exhibited significant abundance changes when compared with the control group. From these, 3 down-regulated proteins were grouped in clusters 2 and 3, while 10 up-regulated ones were grouped in cluster 1 (Figure 8; supplementary excel file 4). The identification of these proteins reveals that OC-based associative memories have an early time sensitive window for protein synthesis prior to the first hour after training.

Operant Conditioning (OC)-Based Associative Memories Possess Particular Molecular Signatures

Memory consolidation may take several days (Squire et al., 2015). During this time, activated cells of neural networks undergo large waves of transcription and translation changes (Borovok et al., 2016; Cavallaro et al., 2002; Monopoli et al., 2011). Two of these major expression waves have been identified in hippocampal cells of rats subjected to a spatial paradigm at 3 and 12 h after conditioning. To examine the protein changes taking place at later time intervals, we analyzed the hippocampal proteome of rats 12 h after conditioning. We identified a total of 170 proteins with statistically significant abundance changes (supplementary excel file 3). Of these, 63 were down-regulated and 107 were up-regulated. Fuzzy c-means clustering analyses grouped 92 of these proteins in cluster 1 and 54 in cluster 2 (Figure 8).

To extract biological meaning from the proteins in this data set, we classified them using the Database for Annotation, Visualization and Integrated Discovery (DAVID) (D. W. Huang et al., 2007). By assigning gene ontology (GO) terms to 166 proteins ($p < 0.05$), we observed that our data was enriched for cytoplasmic, extracellular exosome, nucleus and membrane proteins (supplementary excel file 5). Other terms such as protein complex, post-synaptic density, axon, dendrite and neuron projection were also assigned to proteins in this data set. Further, by analyzing the functional categories of these proteins, we found that the top enriched terms were phosphoprotein, cytoplasm and acetylation ($p < 0.05$; supplementary excel file 5). These results reinforce the notion that PTMs play a central role in the process of memory formation (Routtenberg and Rekart, 2005).

We inferred physical and functional protein-protein associations among these 170 proteins by analyzing them using the STRING database (Szklarczyk et al., 2017); this interaction-prediction tool indicated that only a small set of proteins identified in this time interval did not display associations (Figure 9A). We further subjected the protein network generated by STRING to clustering with overlapping neighborhood expansion (ClusterONE), an algorithm which predicts protein complexes from protein-protein interaction data (Nepusz et al., 2012). This analysis generated a total of 6 protein clusters ($p < 0.05$), which might represent genuine protein complexes in cells (Figure 9B). Cluster V had the largest number of proteins with a total of 8 members. Proteins of the 14-3-3 protein family, which have been associated with the regulation of the cytoskeleton and the formation of new associative memories in the hippocampus (Angrand et al., 2006; Qiao et al., 2014), were enriched in cluster III and IV. In cluster IV, members of the 14-3-3 protein family were shown to interact with member of the cytoskeleton such as tubulin. These results are consistent with the pivotal role of neural circuitry rearrangements underlying the storage of new information in the brain (Diana et al., 2007; Lamprecht, 2011).

We also compared the identity of these 170 differentially regulated proteins at 12 h after OC to a protein data set obtained from the hippocampi of rats at 12 h after SMT (Monopoli et al., 2011). We hypothesized that there would be little overlap between these sets due to the distinct nature of these memories. (Monopoli et al., 2011) identified a total of 18 differentially regulated proteins at 12 h after subjecting rats to SMT. Albeit we have identified 17 of these proteins in the

total hippocampal proteome, we only obtained quantitative information for 11 of them after statistical filters. By comparing the data sets, we identify a single regulated protein being share by the two assays, namely protein/nucleic acid deglycase DJ-1 (Park7). This observation suggests that OC-based associative memories possess particular molecular signatures, in spite of the fact that memory trace from different kinds of behavioral paradigms share common molecular mechanisms.

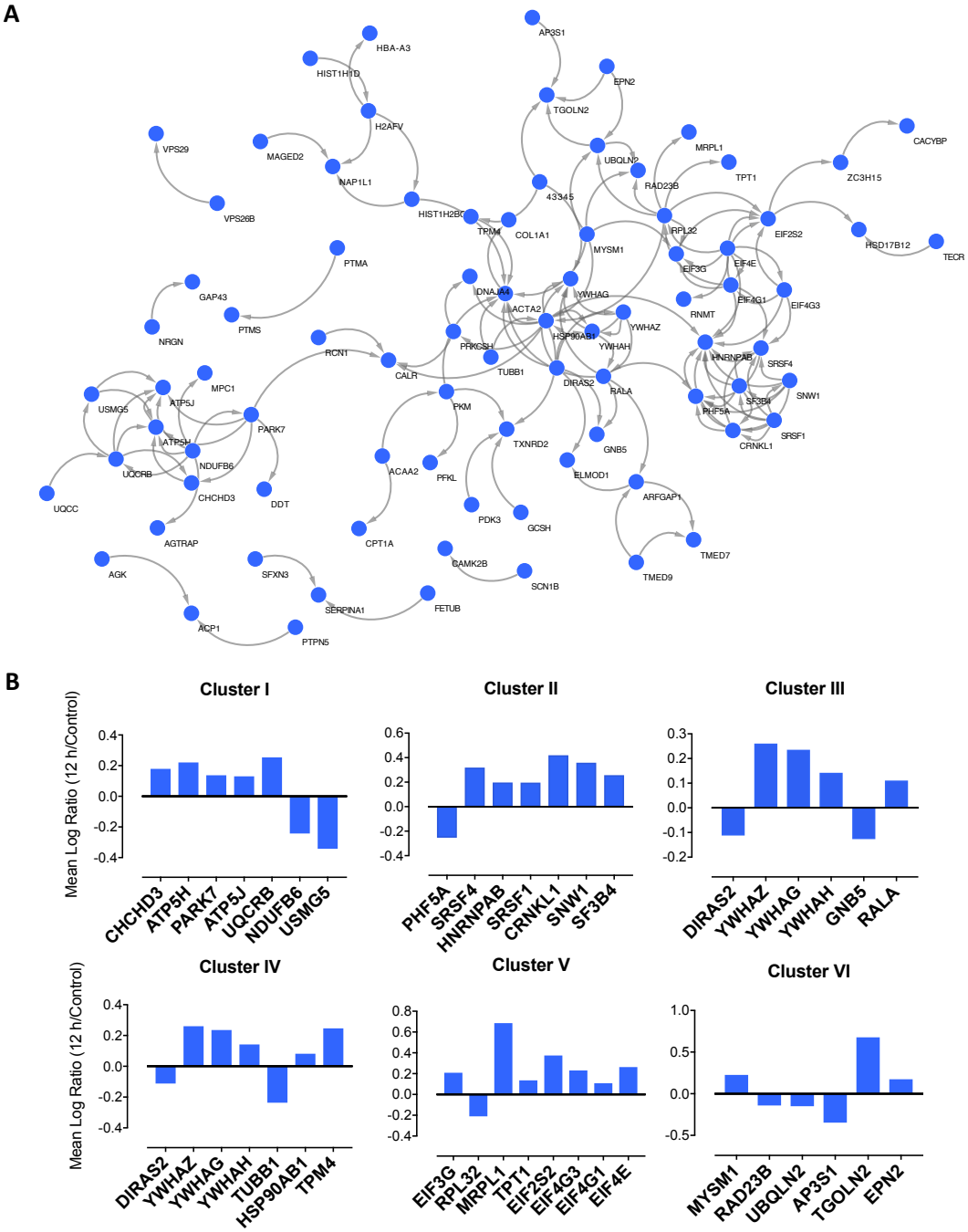

FIGURE 9 I Putative Protein-Protein Associations Among Significantly Regulated Proteins at 12 h After Conditioning. (A) Protein network displaying possible protein-protein interactions among significantly regulated proteins at 12 h after OC. (B) Clusters displaying possible protein complexes ($p < 0.05$) among proteins differentially expressed 12 h after training.

Increase of *De Novo* Protein Synthesis After a Recall Session

Compared to the two first time points, the proteome analysis of operant conditioned rats subjected to a recall session displayed the largest number of proteins exhibiting significant abundance changes. We identified a total of 275 differentially regulated proteins at this time interval, of which 60% were up-regulated. These results strongly suggest that some of these proteins are associated with the process of retrieval itself, since earlier studies have shown that the majority of molecules are down-regulated at 24 h after conditioning. (Monopoli et al., 2011), for instance, showed that the number of proteins being up-regulated at this point decreases when compared to earlier time intervals. (M. M. Ryan et al., 2012), likewise, measured mRNA levels at 24 h after LTP induction in the hippocampi of rats and observed that 68% of the transcripts exhibiting significant abundance changes were down-regulated.

To gain insight into the roles of the regulated proteins after the recall session, we assigned them GO terms using DAVID (D. W. Huang et al., 2007). Cellular component (CC) analysis revealed enrichments for the terms cytoplasm, extracellular exosome, membrane and mitochondrion ($p < 0.05$; supplementary excel file 6). Proteins associated with axon, neuron projection, PSD and synaptic vesicle were also detected in the CC analysis, but they were represented to a lower degree. Notably, proteasome complex was also a term amongst the CC analysis. Proteins classified under this term are members of the ubiquitin proteasome system, which has been associated with the process of memory reconsolidation (S.-H. Lee et al., 2008). Using DAVID, we also carried out enrichment analysis for functional categories (FC) and KEGG pathway. Interestingly, the top hit terms of FC were phosphorylation, acetylation and methylation ($p < 0.05$; supplementary excel file 6). These results show that PTMs may be playing important roles in shaping neural networks after recall of an operant conditioning behavior. KEGG pathway analysis grouped synaptotagmin 1 (Styt1), complexin-2 (Cplx2) and ras-related protein Rab-3A (Rab3A) under synaptic vesicle cycle. Notably, all of these proteins are linked with exocytosis and

were shown to be up-regulated (C.-C. Huang et al., 2011; McMahon et al., 1995; Tucker and Chapman, 2002).

Next, we used the STRING database to infer possible protein-protein interactions among the differentially regulated proteins after the recall session (Figure 10A). Subsequently, we subjected this network to the clustering algorithm of ClusterONE to predict protein complexes (Figure 10B). ClusterONE analysis generated a total of 11 clusters ($p < 0.05$). Among these putative protein complexes, we detected members of the eukaryotic initiation factors (eIFs). Notably, it has been known that eIFs participate in the initiation phase of *de novo* protein synthesis by recruiting mRNAs or stabilizing the ribosomal pre-initiation complex, which is comprised of the 40S ribosomal subunit and the methionyl-transfer RNA (Jackson et al., 2010).

Translation initiation process is orchestrated by a large range of eIFs such as eIF2, eIF3 and eIF4. Interestingly, among the proteins with significant abundance changes after the recall session, we identified a large number of eIFs isoforms. While some of these were up-regulated, others were down-regulated. This apparent discrepancy in abundance levels can be accounted for by site specificity. For instance, some regions of the hippocampus may require protein synthesis and induces the synthesis of specific eIFs; in other sections, the opposite may happen. Notably, among the up-regulated proteins in this dataset, we identified the eIF2β. This is an isoform of eIF2, which helps to assembly of the 43S pre-initiation complex. These observations provide further support to the hypothesis that *de novo* protein synthesis is taking place after the recall session and is related to memory reconsolidation.

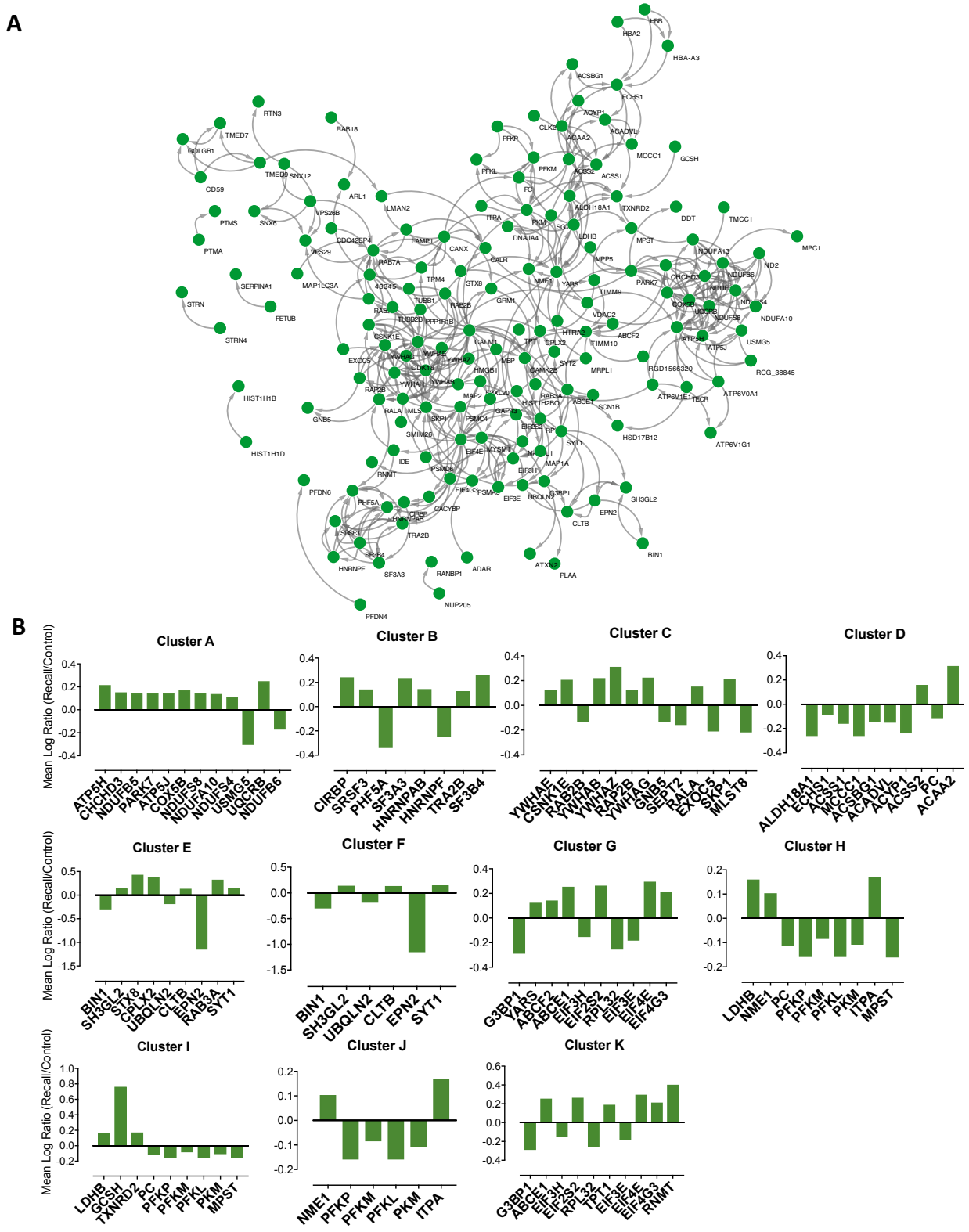

FIGURE 10 I Putative Protein-Protein Associations Among Significantly Regulated Proteins After the Recall Session. (A) Protein network exhibiting putative protein-protein interactions among significantly

regulated proteins after the recall session that took place 24 h after training. (B) Clusters displaying possible protein complexes ($p < 0.05$) among proteins differentially expressed after behavioral retrieval.

Quantitative Hippocampal Phosphoproteome

Phosphorylation plays a significant role in the process of memory consolidation and may also be relevant to plasticity mechanisms underlying behavior retrieval, since this PTM was the top enrichment term for cellular component in both the 12 h and the recall protein data sets. Nevertheless, no large-scale proteomic assay has been performed to understand the role of phosphorylation during memory formation and retrieval. To address this question in operant conditioned rats, we combined iTRAQ labeled peptides once more and enriched for phospho-modified analytes using TiO_2 beads, a step required prior to LC-MS/MS analysis due to the substoichiometric levels of PTMs in cells.

Using a single-shot strategy, we identified 568 phosphoproteins and extracted iTRAQ intensities of the reporter groups to obtain quantitative information for these phosphopeptides (supplementary excel file 7). After correction for protein abundance, statistical analysis in R showed that 53 phosphopeptides exhibited significant abundance changes throughout the three time points under investigation here ($p < 0.05$; supplementary excel file 8). Interestingly, we did not detect any significantly regulated phosphoprotein at 30 min after training, although we showed that CaMKII was up-regulated at this time point. Conversely, 4 phosphopeptides displayed different abundance changes at 12 h when compared with the control individuals. Among these, we have detected the up-regulation of EIF3G phosphorylation. Notably, (Martineau et al., 2014) demonstrated that EIF3G phosphorylation is required for translation initiation, which is consistent with the notion that hippocampi cells of conditioned rats are synthesizing new proteins at later stages of memory consolidation.

Analysis of the phosphoproteome of rats subjected to a recall session demonstrated the differential regulation 49 phosphopeptides. Surprisingly, 80% of these phosphoproteins were down-regulated. To investigate the role of these proteins in rodent cells, we used the DAVID database to assign terms for cellular component (CC) and functional category (FC) ($p < 0.05$; supplementary excel file 9). Among the overrepresented terms for CC were post-synaptic density, synapse, dendrite and neural cell body. These results re-inforce that such locations are under-

regulation after memory retrieve. Conversely, the top enrichment terms for functional categories were phosphorylation, acetylation, alternative splicing, cytoskeleton, cell projection and intermediate filament. This shows that not only PTMs are important in this time interval, but rearrangement of the cytoskeleton seems to play a vital role upon recall.

RT-qPCR Measurements of mRNAs

Recently, a number of studies have sought to address the question of how mRNA levels are related to their protein counterparts in distinct biological systems. These studies found a high similarity between transcript and protein abundances in steady-state conditions, while highly dynamic cellular states exhibited large differences (Liu et al., 2016). To investigate if mRNA abundance mirrors their protein counterparts during memory consolidation and after recall, we selected a total of 5 targets (supplementary excel file primers) and measured their concentration using real-time quantitative polymerase chain reaction (RT-qPCR). *Gria1* and *Camkll*α protein counterparts did not exhibit significant abundance changes at none of the time interval under study here, but they have been shown to be regulated in other behavioral paradigms such as fear conditioning and spatial memory tasks (M. C. Frey et al., 2009; Lisman et al., 2002). RAB3A, YWHAB and YWHAG, contrary, displayed significant abundance changes at 12 h and/or after recall (supplementary excel file 3).

mRNA measurements established that the levels of only one targeted transcript, namely *Gria1*, was significantly altered (one-way ANOVA, $p < 0.05$; Figure 11). The mRNA of this analyte was up-regulated only at 12 h, although proteomic results showed that the protein counterpart of *Gria1* did not exhibited significant changes in abundance at any of the measured time points. Notably, these RT-qPCR results are in agreement with other large-scale quantitative proteomic and transcriptomic studies (Cheng et al., 2016; R. Lu et al., 2009). In part, this low interdependence can be accounted for by differential translation rates among mRNA species, post-transcriptional regulation of gene expression and protein transport (Liu et al., 2016). Taken together, these results highlight the molecular complexity underlying behavioral assays and the need to dissect molecular events underlying memory at multiple levels.

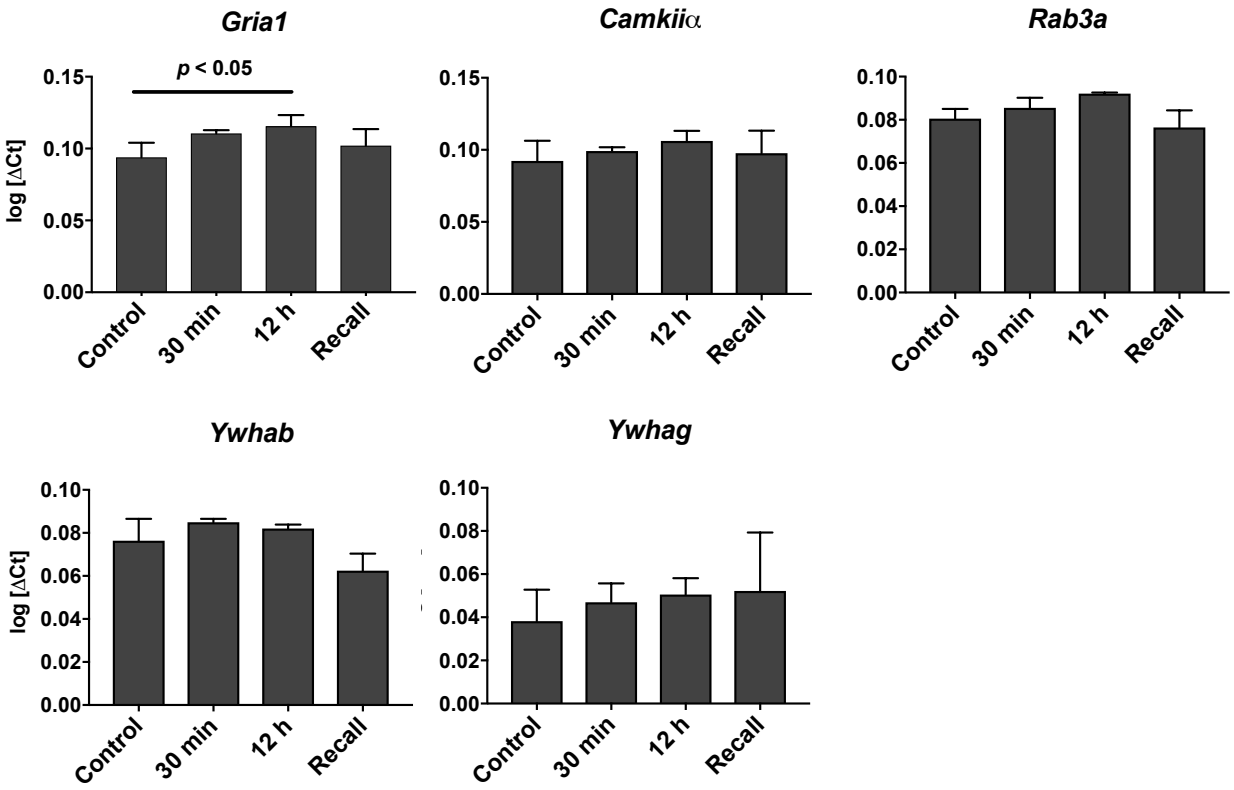

FIGURE 11 I RT-qPCR Measurements of mRNAs. We selected a total of five transcripts and measured their concentration at 30 min, 12 h and after a recall session that took place 24 h after training. *Gria1* and *CamkIIα* protein counterparts did not exhibit significant abundance changes through the aforementioned time intervals, yet *Gria1* was up-regulated at 12 h after conditioning (one-way ANOVA, $p < 0.05$). RAB3A, YWHAB and YWHAG proteins were differentially regulated at least in one of the measured time points; however, their transcripts were not. Hence, RT-qPCR measurements of the selected mRNAs show a low correlation with their protein counterparts.

Discussion

Large-scale transcriptomic and proteomic studies have been carried out in animals subjected to behavioral paradigms to understand the process of memory consolidation in the hippocampus and other regions of the brain. For instance, (Cavallaro et al., 2002) exposed rats to consecutive training sessions in the water maze paradigm, a type of spatial memory task (SMT) and measured mRNA levels at three different time points after it. (Rao-Ruiz et al., 2015), by contrast, quantified hippocampal membrane proteins of fear conditioned (FC) mice at 1 h and 4 h after subjecting these animals to two distinct behavioral protocols: a foot shock was delivered immediately upon placing the animals in the box or was delayed by 3 min. They did not observe proteome changes at 1 h in neither of these experimental groups. At 4 h, however, they identified 164 proteins exhibiting significant abundance changes in animals subjected to the delayed shock protocol and 273 proteins in the immediate shock group.

Interestingly, pharmacological, electrophysiological and genetic approaches have revealed that different forms of behavioral paradigms share the activation of similar molecular and cellular events underlying the retention of newly acquired information. For example, operant and fear conditioning induce the activation of protein kinase A (PKA) and, in its turn, the increase of CREB phosphorylation (Lorenzetti et al., 2008; McGaugh, 2000). As it was mentioned earlier, CREB is a transcription factor known to activate a large number of genes involved in memory consolidation. Nevertheless, global transcript and protein abundance changes vary significantly among these different types of memories. In their assay, for instance, (Rao-Ruiz et al., 2015) demonstrated that less than 20% of all differentially regulated membrane hippocampal proteins identified at 4 h after conditioning were share between the immediate and delayed foot shock groups.

To identify proteins responsible for the consolidation of other types of memories besides aversive and spatial memories, we performed the first temporal quantitative hippocampal proteome and phosphoproteome analyses of operant conditioned rats. After establishing the superior in-depth coverage of the SAX fractionation strategy, we identified a total of 8,951 proteins with at least one unique peptide. We showed that the levels of 465 proteins were significantly altered at three distinct time points – 30 min, 12 h and after a recall session that

took place 24 h after training. Furthermore, enrichment of phosphopeptides using titanium dioxide (TiO_2) beads followed by single-shot analyses in a high-resolution mass spectrometer enabled the identification of 568 phosphoproteins. Of these, 53 exhibited significant abundance changes during the aforementioned time intervals.

At 30 min after conditioning, we identified a total of 20 regulated proteins. These results support the hypothesis of an early time sensitive window for protein synthesis during memory consolidation (Bourtchouladze et al., 1998). In addition, they are consistent with previous experiments which showed that pharmacological inhibition of protein synthesis prior to or immediately after training causes deficits in memory formation in rodents (Bourtchouladze et al., 1998). Further endorsement to this notion comes from the identification of the up-regulation of a subunit of the eukaryotic initiation factor (eIF), namely eukaryotic translation initiation factor 3 subunit G (eIF3g) (Zhou et al., 2008), at this early time-point. EIFs facilitate the synthesis of new proteins in cells and the up-regulation of different subunits have been linked with synaptic plasticity mechanism underlying the process of memory consolidation (Klann and Dever, 2004). In particular, it has been demonstrated that inactivation of *Eif3g* leads to problems in the process of scanning for the start codon of mRNA sequences (Aitken et al., 2016).

Surprisingly, we did not identify any differentially regulated phosphopeptides during the earliest measured time-point. The absence of significant alterations in phosphoprotein at 30 min after conditioning could have resulted from a combination of two factors. First, the method used to isolate phosphopeptides could have depleted species that underwent abundance changes. For instance, (Bodenmiller et al., 2007) employed three different techniques to isolate phosphopeptides – phosphoramidate chemistry, immobilized metal affinity chromatography (IMAC) and TiO_2 beads – from *Drosophila melanogaster* Kc167 cells and analyzed the samples in a mass spectrometer. They found that each isolation strategy enriches for a specific set of phosphopeptides. Accordingly, data sets generated from these methods showed a weak overlap (Bodenmiller et al., 2007). Second, it is possible that phosphoprotein levels may not undergo significant alterations at 30 min after conditioning, but at earlier or later time points. This would occur if, for instance, the activation of protein kinases happened at different time points during the consolidation of newly acquired memories (Izquierdo et al., 2006).

Protein kinases have been shown to play a singular role in memory. During LTP plasticity mechanisms, for instance, depolarization of CA1 cells of the hippocampus through tetanic stimulation leads to the influx of calcium and activation of CaMKII, a protein kinase that plays an important role in LTP. Interestingly, repeated stimulation of CA3 cells leads to the activation of PKA in the CA1 post-synaptic neurons by the increase in cyclic adenosine monophosphate (cAMP). Activated PKA turns on mitogen activated protein kinase (MAPK), which translocates to the nucleus and phosphorylates CREB (Pontes and de Sousa, 2016). Furthermore, repeated tetanic stimulation also leads to the activation of protein kinase C zeta. This protein kinase is required for the persistence of memory, but not for its initial induction (Tsokas et al., 2016).

Among the up-regulated proteins at 30 min after conditioning, we identified a fold-change increase of 0.2 in the β-subunit of CaMKII. Interestingly, previous FC or SMTs behavioral paradigm studies have detected major changes in the expression of the α-subunit of CaMKII (Ahmed and J. U. Frey, 2005b). CaMKII α- and β-subunits are the most abundant isoforms of CaMKII in the brain of vertebrates (Lisman et al., 2002). We quantified the α-subunit at all time points, but its abundance did not change significantly during the course of the experiment. Consistent with these results, quantification of CaMKIIα transcript was not altered when compared with the control group. By contrast, the protein levels of the CaMKIIβ increased significantly as early as 30 min after conditioning. These results suggest that – in addition to global scale variations in the proteome – different memory types also exhibit differences in conserved signaling mechanisms that are shared among them.

Analysis of protein changes at 12 h after exposing rats to OC revealed that 170 proteins exhibited significant abundance changes between subject and control animals. We grouped these proteins into three different clusters that display the temporal behavior of the hippocampal proteome during memory formation and after recall (Figure 8; supplementary excel file 4). Among the 107 up-regulated proteins, we found the isoforms YWHAG, YWHAH and YWHAZ, which are members of the 14-3-3 protein family. In mammals, this protein family possesses a total of seven isoforms and account for 1% of all proteins expressed in the brain. Notably, members of the 14-3-3 protein family have been linked to a variety of cellular processes such as cell cycle, intracellular trafficking, transcriptional control and signal transduction (Berg et

al., 2003). In addition, previous studies showed that they interact with a large array of proteins, ranging from enzymes and protein receptors to cytoskeletal and structural proteins (Kent et al., 2010). Moreover, it has been demonstrated that these proteins play a crucial role during memory formation (Qiao et al., 2014).

Initial evidence for the role of 14-3-3 protein family isoforms in memory came from studies in *Drosophila* flies containing mutations in the gene *leonardo* (*leo*), which encoded one of the isoforms of this protein family. Interestingly, *leo* flies exhibited memory deficits and a reduction in basal synaptic transmission when they were subjected to an olfactory learning paradigm (Skoulakis and Davis, 1996). In mammals, rodents have been used to study alterations in memory formation associated with these proteins. (Qiao et al., 2014), for example, used transgenic mice expressing an inhibitor of the 14-3-3 protein family isoforms in the neurons of the CA3-CA1 regions of the hippocampus to study alterations in memory formation within a FC context. They showed that expression of this protein inhibitor lead to deficits in memory consolidation when animals were retested 24 h after training. Consistent with the involvement of the 14-3-3 protein family in mediating synaptic transmission within cells, (Qiao et al., 2014) established that LTP induction in CA1 cells expressing the inhibitor was eliminated 1 h after high-frequency stimulation. This study, however, was not able to pin point the exact contribution of each isoform to the process of memory formation, since the inhibitor targeted all isoforms of this protein family.

The quantitative temporal analysis of the hippocampal proteome of operant conditioned rats carried out here showed that the isoforms of the 14-3-3 protein family are regulated at distinct time intervals, which suggests that these isoformic species play different roles during the process of memory consolidation and after recall. We established that a fold-change increase of 0.23 of the isoform YWHAG was not accompanied by an up-regulation of in its mRNA counterpart, suggesting that the expression of this protein is regulated post-transcriptionally. Interaction networks containing regulated proteins at 12 h after conditioning indicated that YWHAG, YWHAH and YWHAZ were associated with structural or cytoskeletal proteins such as members of the tubulin family (Figure 9B). These results endorse previous studies and suggest that this protein

family is involved with the morphological changes within hippocampal cells that are linked with the retention of information at later time points (Berg et al., 2003; Qiao et al., 2014).

Interestingly, recent observations have led to the conception that protein degradation might be as important as protein synthesis for the process of memory formation. According with this emerging hypothesis, the breakdown of specific groups of proteins withdraws possible inhibitory constraints on memory consolidation (S.-H. Lee et al., 2008). The degradation of these proteins can occur via three main proteolytic routes – action of proteases such as neurotrypsin, lysosome or the ubiquitin-proteasome system (UPS), which tags proteins for degradation through the transfer of ubiquitin to lysine residues (Fioravante and Byrne, 2011; Mueller et al., 2015). Notably, our data analysis revealed that 37% of the proteins with significant abundance changes at 12 h after conditioning were down-regulated. Yet, we did not detect the up-regulation of any subunits of these protein degradation complexes, suggesting that they are controlled at the levels of activity and/or that they are temporally expressed at different time intervals (X. Wang et al., 2013).

On this note, protein degradation has also been implicated in memory reconsolidation – a process by which information become liable to changes upon behavioral retrieval. (S.-H. Lee et al., 2008) investigated the role of the UPS in memory reconsolidation. They infused β-lactone (i.e a proteasome inhibitor), ANI or both bilaterally in the hippocampus of fear conditioned mice after a recall session. Re-testing animals 24 h later revealed that while ANI infused mice had a significant decrease in fear levels, administration of β-lactone alone did not affect fear levels. Surprisingly, co-administration of β-lactone reversed the amnesic effects of ANI. (S.-H. Lee et al., 2008) concluded that the proteasome inhibitor destabilized the reactivated memories, yet protein synthesis is still required for the re-stabilization of consolidated memory. That is, consolidated memories could be reconfigured during recalls by degradation and new protein synthesis.

In order to access the role of protein degradation during memory retrieval in operant conditioned rats, we performed cellular component analysis of the regulated proteins after the recall session. Results revealed an enrichment for proteins associated with ubiquitin proteasome system. Notably, some ubiquitin proteasome system components such as proteasome 26S

subunit, non-ATPase, 6 (Psmd6) and ubiquitin-like modifier-activating enzyme 2 (Uba2) and proteasome subunit alpha type-6 (Psma6) had a fold-change decrease of 0.13, 0.23 and 0.15 respectively, while other proteins like ubiquitin-conjugating enzyme E2 N (Ube2n) and 26S proteasome regulatory subunit 6B (Psmc4) were up-regulated. A plausible explanation for these observations is that within neural networks in the hippocampus, different components of the proteasome have distinct specificities and activities for substrates (X. Wang et al., 2013).

Recently, (Jarome et al., 2016) demonstrated that CaMKII is critical for an increase in proteasome activity in the amygdala following memory recall. First, they showed that CaMKII inhibition *in vitro* reversed the increase of proteasome activity in animals subjected to a recall session after FC training. Second, using *in vivo* blockage of this kinase, they abolished proteolytic activity following recall. These results uncovered another function of CaMKII in shaping newly acquired external information – control of proteasome activity during memory reconsolidation. Here, we have also detected a fold-change increase of 0.17 of CAMKIIβ after behavior recall, as well as an increase in proteins that are a part of the proteasome system such as the ones mentioned above. These observations reinforce our claim that memory reconsolidation is taking place in the hippocampus of operant conditioned rats.

Notably, the aforementioned results stand in contrast to earlier studies, which did not detect clear evidence for memory reconsolidation in operant conditioned animals (Exton-McGuinness et al., 2014; Hernandez and Kelley, 2004; Tronson and Taylor, 2007). The apparent absence of memory reconsolidation could have resulted from the adopted training protocol – subjecting rodents to 10 consecutive training sessions prior to retrieval might have masked processes associated with memory reconsolidation. To be more precise, several studies have shown that hippocampal dependent memories last for a couple of days. Subsequently, this information is transferred to regions of the cortex for long-term storage (Lesburguères et al., 2011). Consistent with this view, (Milekic and Alberini, 2002) trained rats in FC and subjected these animals to recall sessions at different time points after conditioning – 2, 7, 14 and 28 days later. They found that animals became vulnerable to memory disruptions through the administrations of protein inhibitors only at early time points, showing that the process of reconsolidation can only occur in newly acquired memories. The results of (Milekic and Alberini,

2002) support behavioral assays in which memory reconsolidation was detected – in the majority of these studies, animals were subjected to at most 3 training sessions. Taking these observations into perspective, we reason that the inability to detect memory reconsolidation in operant conditioned animals in early studies is a result of the training protocols performed in these previous experiments (Hernandez and Kelley, 2004; Tronson and Taylor, 2007).

After the recall session, we also identified a fold-change increase of 0.4 in the levels of syntaxin 8 (Stx8). This protein is a member of the Q-soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (Q-SNARE) family that mediates vesicle trafficking inside cells (Hong, 2005). Knockdown and overexpression assays showed that Stx8 facilitates tropomyosin receptor kinase A (TrKA) transport from the Golgi to the plasma membrane (B. Chen et al., 2014). Activated TrkA induces downstream signal transduction cascades including phosphoinositide-3–protein kinase B/Akt (PI3K-PKB/Akt) and Ras/mitogen-activated protein kinase (MAPK) pathways, which are both involved in LTP mechanisms of synaptic plasticity in the hippocampus (Giovannini et al., 2001; Sui et al., 2008). Interestingly, (Cavallaro et al., 2002) showed that, in STM subjected rats, Stx8 is up-regulated at 1 h after the last training session, but not at 12 h nor 24 h after training. This indicates that this protein might be involved in memory recall processes and not in the formation of new memories. Given that (Cavallaro et al., 2002) subjected animals to 3 training sessions, their measurements of *Stx8* transcripts might be associated with the function of Stx8 in memory recall events.

In summary, we provide the first temporal large-scale proteome and phosphoproteome data set of rats subjected to an operant conditioning task by employing SAX as a pre-fractionation strategy prior to LC-MS/MS. We revealed the identify of proteins differentially regulated as early as 30 min after training, including the β-subunit CaMKII. At later time points, we showed that proteins associated with the regulation of the cytoskeleton such as members of the 14-3-3 protein family are important in the rearrangement of neural circuitries in the hippocampus. Interestingly, regulation of these proteins was not accompanied by changes in their mRNA counterparts, reveling a low interdependence between these macromolecules. Moreover, the identification of differentially regulated proteins of the ubiquitin-proteasome system (UPS) after a recall session provides strong evidence for memory reconsolidation in OC, wherein we

hypothesized that this process has not been fully observed earlier due to the behavioral protocols usually used in conditioning animals. We hope that the proteome data set of operant conditioned rats presented here will help to bridge the gap in the understanding of the different memory types, as well as disease-associated phenotypes.

Materials and Methods

Ethical Committee

All experimental procedures performed here were approved by the Ethics Committee of the Institute of Biological Sciences of the University of Brasilia (document identification number: 23106.075295/2016-61).

Animals and Behavioral Protocol

Three-month-old Wistar female rats (n = 12) were maintained in a 12/12 h light/dark cycle with water and food *ad libitum*, being handled every day for two weeks prior to any experimental procedure. Animals were then water restricted for 18 h throughout the experiment. Their body weight was checked daily to ensure it did not drop below 80% of its initial weight. Following handling, water restricted animals were placed in a standard Skinner box (Insight Ltda, São Paulo, Brazil) for two days of habituation. In these 20 min habituation sessions, animals were put in the box and water was released at random from time to time. Habituated female rats were then returned to the Skinner box and manually trained in a single session. The training consisted on releasing water as animals got progressively closer to the lever. Eventually, these rats learned that pressing the lever in the box would result in water release. The criterion for behavior acquisition was set at 15 lever presses in a row. Moreover, animals (n = 3) of the recall group were placed once again in the box one day after training to retrieve the behavior. The criterion in the recall session was set at 50 lever presses in a row. Female rats (n = 3) of the control group did not undergo training, but went through the habituation steps described earlier and were also placed in the box for 20 min in the day of the training session.

Tissue Dissection

Operant conditioned female rats were euthanized at 30 min, 12 h and 30 min after a recall session that took place 24 h after conditioning. Control animals were euthanized 30 min after leaving the Skinner box. Their hippocampi were anatomically dissected under 3 min, washed in iced-cold phosphate-buffered saline (PBS) and flash-frozen in liquid nitrogen. Next, those frozen tissues were ground using a pestle and separated in two distinct microtubes. This step was

performed to separate samples for the proteomic and RT-qPCR assays, which require different reagents for the extraction of proteins and mRNAs, respectively.

Filter-aided Sample Digestion

The digestion of proteins into peptides was performed as previously described in (Wisniewski et al., 2009), with some modifications. Briefly, ground frozen hippocampal samples were homogenized in 0.3 mL of 4% (w/v) sodium dodecyl sulfate (SDS), 0.02 M triethylammonium bicarbonate (TEAB), 0.1 M dithiothreitol (DTT), phosphatase inhibitor cocktail (Roche) and pH 7.9. Tubes were then incubated for 10 min at 90 ˚C and sonicated to shear DNA and RNA molecules which might affect digestion efficiency. Subsequently, samples were centrifuged at 16,000 *g* for 15 min at room temperature. The supernatant was saved and protein concentration was measured in all conditions using the Qubit Protein Assay Kit (Thermo Fisher Scientific).

Aliquots containing 100 µg of lysed proteins were mixed with 0.2 mL of urea buffer (UB) – 8 M urea, 0.02 M TEAB and pH 8.5 – in Vivacon 500 (Sartorius) with a nominal cut off of 30KDa. In order to perform the comparison between SCX and SAX pre-fractionation strategies, we digested 500 µg of protein lysates and made adjustments to the volume of the buffer solutions of the steps described below. Vivacon devices were then centrifuged at 10,000 *g* for 15 min at 20 ˚C – all the following centrifugations were performed at this temperature, unless otherwise stated. Samples were washed with 0.2 mL of UB and centrifuged. Subsequently, 0.1 mL of UB containing iodoacetamide to a final concentration of 0.05 M was added to the samples and the Vivacon devices were incubated for 20 min in the dark. Samples in the filtration units were centrifuged, washed with 0.2 mL of UB and re-centrifuged. Concentrated samples in the Vivacon devices were buffer exchanged by washing twice with 0.1 mL of digestion buffer (DB; 0.02 M TEAB and pH 7.9) as described above. These samples were then trypsin-digested in DB with trypsin for 12 h at 37 ˚C with a concentration ratio of 1:100 (protease:sample). Following proteolytic digestion, the samples were collected by centrifugation. The filter units were rinsed with DB and centrifuged one more time. The proteolytic reaction was halted by the addition of

trifluoroacetic acid (TFA) to the samples to a final concentration of 0.5%. Finally, the yield of each digestion was measured with the Qubit Protein Assay Kit.

Labeling the Samples with Multiplex Reagents

Aliquots of 50 µg of each replicate was vacuum dried in a Speedvac and the labeling of the conditions was carried out with the iTRAQ reagent kit (AB Sciex, Framingham, MA) according with the manufacturer's instructions. The labeling was performed as such – control (tag 114), 30 min (tag 115), 12 h (tag 116), recall (tag 117). The four isobaric tags of each biological replicate were mixed in a ratio of 1:1:1:1, desalted and analyzed in a 90-min gradient via LC-MS/MS to verify the efficiency of the labeling reactions and the ratio between the different tags.

Offline Anion Exchange Based Fractionation with Salt Elution

Offline pre-fractionation of peptides was performed according with (Rappsilber et al., 2007), with some minor modifications. Briefly, strong anion exchange (SAX) stop-and-go extraction tips (StageTips) were made in-house by cutting 3M Empore Anion Exchange disks with a cutter and stacking the membranes into a 200 µL pipette tip. A plastic ring was set around the StageTips and they were positioned into 2 mL microtubes. The membranes were activated by loading the pipette tips with 0.1 mL of 100% methanol and centrifuging them – all the centrifugations set at 1,000 *g* and the entire procedure was performed at room temperature. Activated membranes were subjected to a number of washes by loading and centrifuging the tips. First, they were washed with 0.1 mL of 0.1% ammonium hydroxide (NH_4OH) and 80% acetonitrile (ACN); second, 0.1 mL of 0.1% NH_4OH; and third, 0.1 mL of 20% ACN, 0.1% NH_4OH and 2 M ammonium acetate (NH_4AcO). Finally, the membranes were washed twice with a solution of 0.1% NH_4OH. Once those steps were performed, the samples were loaded into the StageTips.

The four different chemically labeled conditions were mixed again in a ratio of 1:1:1:1 to a total of 30 µg of sample per biological replicate – in the experiment done to benchmark the two pre-fractionation strategies, aliquots of 30 µg were also used, yet the samples were unlabeled and came from the sample proteolytic reaction. The peptides were then vacuum dried

in a Speedvac, resuspended in 0.1 mL of 0.5% NH_4OH and loaded into the StageTips. The pipette tips were centrifuged and the flow through was collected in a clean microtube. The fractions were eluted with 0.03 mL solutions of 20% ACN, 0.1% NH_4OH and an increasing concentration of NH_4AcO – 0.015 to 3 M. Finally, the 12 fractions of each replicate, including the flow through, were desalted and analyzed by LC-MS/MS. In the experiment to benchmark SAX with SCX, only 7 fractions were analyzed per replicate.

Offline Cation Exchange Based Fractionation with Salt Elution

Offline strong cation exchange (SCX) based fractionation was done with 30 µg of proteolytic digest per technical replicate. These samples came from the same enzymatic reaction used in the SAX fractionation experiments – this was done intentionally to decrease any variation arising from distinct proteolytic cleavage reactions. The fractionation was performed according to (Rappsilber et al., 2007), with minor modifications. StageTips were made in-house as described above, with 3M Empore Cation Exchange disks, and all the centrifugation were carried out at 1,000 *g* at room temperature.

In order to activate the membranes, we added 0.1 mL of 100% ethanol to the tips and centrifuged them. Next, we placed an 0.1 mL solution of 0.5% acetic acid (AcOH) and 80% ACN, followed by a centrifugation. Subsequently, the membranes were equilibrated with 0.1 mL of 0.5% acetic acid solution. We added 0.1 mL of 20% ACN, 0.5% AcOH and 2 M NH_4AcO to the tips and centrifuged them. Before placing the samples in the pipette tips, we washed the membranes twice with a 0.1 mL solution of 0.5% AcOH. Vacum dried peptides were resuspended in 0.1 mL of 0.5% TFA and loaded into the StageTips. Fractions were then eluted with 0.03 mL solutions of 20% ACN, 0.5% AcOH and an increasing concentration of NH_4AcO – 0.15 to 3 M. The 7 fractions of each replicate, including the flow through, were desalted using C_{18} StageTips (see below) and analyzed by LC-MS/MS.

Enrichment of Phosphopeptides Using TiO_2 Beads

Phosphopeptide enrichment was performed with iTRAQ labeled peptides according with (Thingholm et al., 2008). Briefly, the chemical labeled experimental and control conditions were

combined in a ratio of 1:1:1:1. Starting with a total concentration of 100 µg, each biological replicate was diluted in 80% acetonitrile, 5% TFA and 1 M glycolic acid. Next, titanium dioxide (TiO_2) beads of 5 µm in diameter (GL Science, Japan) were added to the samples in a ratio of 0.6 mg/100 µg (bead/peptide) and the biological replicates were incubated for 20 min at room temperature. After that, the samples were spun down and the supernatant was stored. The beads were washed twice, first with a solution of 80% ACN and 1% TFA and then with 10% ACN and 0.1% TFA. The phosphopeptides were eluted from the beads with a solution of 1.5% NH_4OH of pH 11. The samples were then vacuum dried and cleaned up with C_{18} StageTips.

Desalting in C_{18} StageTips

Prior to LC-MS/MS, all the samples were desalted according with (Rappsilber et al., 2007), with some minor modifications. First, StageTips were made in-house as previously described here using 3M Empore Exchange disks containing C_{18} bonded silica. Next, activation and equilibration of the membranes were performed as follows: we added 0.1 mL of 100% ethanol to each StageTip and centrifuged them. Subsequently, we placed 0.1 mL of a solution containing 0.5% AcOH and 80% ACN into the tips and centrifuged them. Finally, 0.1 mL of 0.5% AcOH was added to the C_{18} exchange disks and they were centrifuged; this step was repeated one more time.

Once the StageTips had been activated and equilibrated, the vacuum dried samples were resuspended in a solution of 1% TFA and placed into the tips. Next, the membranes were centrifuged. Before eluting the peptides, the membranes were washed twice with 0.1 mL of 0.5% AcOH. After this step, the samples were eluted with a solution containing 0.5% AcOH with an increasing concentration of acetonitrile – 25, 50 and 100%. The samples were dried in a Speedvac and analyzed via LC-MS/MS.

Liquid Chromatographic and Mass Spectrometry

All the samples were analyzed by a DIONEX 3000 nanoUPLC (Thermo Scientific) system coupled to an Orbitrap Elite mass spectrometer (Thermo Scientific). Briefly, we resuspended vacuum dried samples in a solution of 0.1% formic acid. Then, peptides were loaded into a Reprosil-Pur 120 C18-AQ in-house packed trap column (5 µm particle size, 5.0 cm length, 100 µm

inner diameter, 360 µm outer diameter). The trap column was washed for 5 min with solvent A (0.1% formic acid, 2% ACN). Washed peptides were eluted into a 35 cm long (75 µm inner diameter) homemade silica column packed with 3 µm C_{18} material (Dr. Maisch GmbH). The peptides were eluted from the column using a gradient of buffer B (0,1% formic acid and 100% ACN) from 10 to 35% for 155 min, from 35 to 90% for 15 min and 90% for 5 min at a flow rate of 230 nl/min. After each run, the column was washed and re-equilibrated. Mass spectra data were acquired in a data-dependent (DDA) mode, wherein each MS scan (350 – 1650 m/z at a resolution of 120,000 FWHM) was followed by MS/MS scan (at a resolution 15,000 FWHM) with a dynamic exclusion of 30 s. In the benchmarking assay of SAX and SCX, we fragmented the top 15 most intense peptides by higher energy collisional dissociation (HCD) with a normalized collision energy (NCE) of 35%. In the phosphoproteome experiment, only the top 12 most intense labeled ions were fragmented by HCD with a NCE of 35%. For the labeled iTRAQ strong anion fractions, we fragmented the top 20 most intense peptides by HCD with a NCE of 37%.

Computational and Statistical Data Analysis

Raw files of the LC-MS/MS runs have been deposited in the ProteomeXchange Consortium through the PRIDE (Vizcaíno et al., 2016) partner repository with the data set identifier PXD009782. These raw files were analyzed in Proteome Discover v2.1. Data were searched against the *Rattus norvegicus* database (Proteome ID: UP000002494) from Uniprot/SWISS-PROT with a precursor mass tolerance of 10 ppm and a fragment mass tolerance of 0.05 Da. Searches were performed using MS Amanda 2.0, as well as Sequest HT, and conducted with methionine oxidation, deamidation, acetylation and iTRAQ labeling as variable modifications. Cysteine carbamidomethylation was set as a fixed modification. In the assay that we benchmarked SAX to SCX, iTRAQ labeling was not set as a variable modification and only Sequest HT was used as a searching engine. In the phophosproteome experiment, phosphorylation was also set as a variable modification. Data were filtered to a 1% FDR using the percolator function of Proteome Discover, unique quantification and only high confident peptides were used in further processing. Phosphorylated peptides were filtered according to TODO for the localization of the modifications.

Quantified peptide-spectrum matches from enriched and non-enriched fractions were each log2-transformed and normalized by the median before combining the fractions. Multiple peptide-spectrum matches were summarized by taking their mean and scaled to 0 level after removing outliers by Grubb's test (min. 5 values, threshold p=0.05). Proteins quantifications were obtained by summarization of the peptides following the same method as for peptide-spectrum matches. Phosphorylated peptides were adjusted by subtraction of protein expressions from identical samples. Statistical tests were performed for proteins and phosphorylated peptides separately. Differentially regulated features were determined by applying LIMMA and rank product test and correction for multiple testing according to (Schwämmle et al., 2013). Parameters for fuzzy c-means clustering of proteins were obtained using the method in (Schwämmle and Jensen, 2010), providing a cluster number of three when using the minimum centroid distance. Proteins with a membership value below 0.5 were assumed not to belong to any cluster.

Real Time PCR Measurements

RNA from the different conditions were extracted in parallel using the TRIzol reagent (Invitrogen) according with the manufacturer's instructions. In short, 1 mL of TRIzol reagent was added to the tissue samples, which were subsequently homogenized in an in-house motor-driven homogenizer for 5 min. Due to the high fat content, the lysates were centrifuged at 12,000 *g* for 5 min at 4 ˚C. The supernatants were transferred to a new tube and they were incubated for 5 min at room temperature. Next, 0.2 mL of chloroform was added to the mixture, tubes were vortexed and incubated for 5 min at room temperature. Samples were centrifuged at 12,000 *g* for 15 min at 4 ˚C, and the colorless aqueous phase containing the RNA molecules was transferred to a new tube. RNA was precipitated from solution by adding 0.5 mL of isopropanol and incubating the samples for 10 min at room temperature. Finally, samples were centrifuged at 12,000 g for 10 min at 4 ˚C and the supernatant was discarded. The RNA pellet was allowed to air-dry and stored at -20 ˚C.

RNA samples were suspended in Tris-EDTA (TE) buffer (10 mM Tris-HCl, 1 mM EDTA, and pH 8) and their concentration were measured using NanoDrop (Thermo Scientific). In order to

remove possible DNA contaminants from the samples, DNA was digested with RQ1 RNase-Free DNase (Promega), according to manufacturer's instructions. DNase treated mRNA samples were used to synthesized cDNA using the High-Capacity cDNA Reverse Transcription Kit (Thermo Scientific). 2 μg of sample RNA were used in reverse transcription reactions as described in the manufacturer's protocols.

Before performing the RT-qPCR measurements, we conducted amplification reactions of the targeted transcripts – *Gria1*, *CamkII*α, *Rab3a*, *Ywhab*, *Ywhag* and *Gapdh* (endogenous reference) – and visualized the amplicons in an 2% agarose gel. Each reaction generated a unique band in the gel which matched with its expected molecular weight. We have also performed dilution curves to measure the efficiency of the primers, showing that they were all close to 100% (Supplementary Table XXX). These dilution curve assays and the RT-qPCR experiments themselves were performed with PowerUp SYBR Green Master Mix (Thermo Scientific) in a StepOnePlus Real-Time PCR System (Applied Biosystems), wherein the cycling conditions were: 95 ˚C for 20 s followed by 45 cycles of 95 ˚C for 3 s, 53 ˚C for 10 s and 60 ˚C for 30 s. *Gapdh* and *CamkIII*α were the only transcripts that had the same annealing and extension temperatures: 60 ˚C for 30 s. In addition, melting curves were performed to confirm PCR products. All the statistical analyses of this data set were carried out in GraphPad Prism using One-Way ANOVA.

References

Abel, T., Nguyen, P.V., Barad, M., Deuel, T.A., Kandel, E.R., Bourtchouladze, R., 1997a. Genetic demonstration of a role for PKA in the late phase of LTP and in hippocampus-based long-term memory. Cell 88, 615–626.

Abel, T., Nguyen, P.V., Barad, M., Deuel, T.A., Kandel, E.R., Bourtchouladze, R., 1997b. Genetic demonstration of a role for PKA in the late phase of LTP and in hippocampus-based long-term memory. Cell 88, 615–626.

Ahmed, T., Frey, J.U., 2005a. Plasticity-specific phosphorylation of CaMKII, MAP-kinases and CREB during late-LTP in rat hippocampal slices in vitro. Neuropharmacology 49, 477–492. doi:10.1016/j.neuropharm.2005.04.018

Ahmed, T., Frey, J.U., 2005b. Plasticity-specific phosphorylation of CaMKII, MAP-kinases and CREB during late-LTP in rat hippocampal slices in vitro. Neuropharmacology 49, 477–492. doi:10.1016/j.neuropharm.2005.04.018

Aitken, C.E., Beznosková, P., Vlčkova, V., Chiu, W.-L., Zhou, F., Valášek, L.S., Hinnebusch, A.G.,

Lorsch, J.R., 2016. Eukaryotic translation initiation factor 3 plays distinct roles at the mRNA entry and exit channels of the ribosomal preinitiation complex. Elife 5. doi:10.7554/eLife.20934

Altelaar, A.F.M., Heck, A.J.R., 2012. Trends in ultrasensitive proteomics. Curr Opin Chem Biol 16, 206–213. doi:10.1016/j.cbpa.2011.12.011

Anderson, N.G., Anderson, N.L., 1996. Twenty years of two-dimensional electrophoresis: past, present and future. Electrophoresis 17, 443–453. doi:10.1002/elps.1150170303

Angrand, P.-O., Segura, I., Völkel, P., Ghidelli, S., Terry, R., Brajenovic, M., Vintersten, K., Klein, R., Superti-Furga, G., Drewes, G., Kuster, B., Bouwmeester, T., Acker-Palmer, A., 2006. Transgenic Mouse Proteomics Identifies New 14-3-3-associated Proteins Involved in Cytoskeletal Rearrangements and Cell Signaling. Mol. Cell Proteomics 5, 2211–2227. doi:10.1074/mcp.M600147-MCP200

Azimifar, S.B., Nagaraj, N., Cox, J., Mann, M., 2014. Cell-type-resolved quantitative proteomics of murine liver. Cell Metab. 20, 1076–1087. doi:10.1016/j.cmet.2014.11.002

Bantscheff, M., Boesche, M., Eberhard, D., Matthieson, T., Sweetman, G., Kuster, B., 2008. Robust and sensitive iTRAQ quantification on an LTQ Orbitrap mass spectrometer. Mol. Cell Proteomics 7, 1702–1713. doi:10.1074/mcp.M800029-MCP200

Bantscheff, M., Lemeer, S., Savitski, M.M., Kuster, B., 2012. Quantitative mass spectrometry in proteomics: critical review update from 2007 to the present. Anal Bioanal Chem 404, 939–965. doi:10.1007/s00216-012-6203-4

Bantscheff, M., Schirle, M., Sweetman, G., Rick, J., Kuster, B., 2007. Quantitative mass spectrometry in proteomics: a critical review. Anal Bioanal Chem 389, 1017–1031. doi:10.1007/s00216-007-1486-6

Beck, S., Michalski, A., Raether, O., Lubeck, M., Kaspar, S., Goedecke, N., Baessmann, C., Hornburg, D., Meier, F., Paron, I., Kulak, N.A., Cox, J., Mann, M., 2015. The Impact II, a Very High-Resolution Quadrupole Time-of-Flight Instrument (QTOF) for Deep Shotgun Proteomics. Mol. Cell Proteomics 14, 2014–2029. doi:10.1074/mcp.M114.047407

Berg, D., Holzmann, C., Riess, O., 2003. 14-3-3 proteins in the nervous system. Nat. Rev. Neurosci. 4, 752–762. doi:10.1038/nrn1197

Bliss, T.V., Collingridge, G.L., 1993. A synaptic model of memory: long-term potentiation in the hippocampus. Nature 361, 31–39. doi:10.1038/361031a0

Bodenmiller, B., Mueller, L.N., Mueller, M., Domon, B., Aebersold, R., 2007. Reproducible isolation of distinct, overlapping segments of the phosphoproteome. Nat. Methods 4, 231–237. doi:10.1038/nmeth1005

Boersema, P.J., Geiger, T., Wisniewski, J.R., Mann, M., 2013. Quantification of the N-glycosylated secretome by super-SILAC during breast cancer progression and in human blood samples. Mol. Cell Proteomics 12, 158–171. doi:10.1074/mcp.M112.023614

Borovok, N., Nesher, E., Levin, Y., Reichenstein, M., Pinhasov, A., Michaelevski, I., 2016. Dynamics

of Hippocampal Protein Expression During Long-term Spatial Memory Formation. Mol. Cell Proteomics 15, 523–541. doi:10.1074/mcp.M115.051318

Bourtchouladze, R., Abel, T., Berman, N., Gordon, R., Lapidus, K., Kandel, E.R., 1998. Different training procedures recruit either one or two critical periods for contextual memory consolidation, each of which requires protein synthesis and PKA. Learning & Memory 5, 365–374.

Castillo, P.E., 2012. Presynaptic LTP and LTD of excitatory and inhibitory synapses. Cold Spring Harb Perspect Biol 4. doi:10.1101/cshperspect.a005728

Cavallaro, S., D'Agata, V., Manickam, P., Dufour, F., Alkon, D.L., 2002. Memory-specific temporal profiles of gene expression in the hippocampus. Proc. Natl. Acad. Sci. U.S.A. 99, 16279–16284. doi:10.1073/pnas.242597199

Chen, B., Zhao, L., Li, X., Ji, Y.-S., Li, N., Xu, X.-F., Chen, Z.-Y., 2014. Syntaxin 8 modulates the post-synthetic trafficking of the TrkA receptor and inflammatory pain transmission. J. Biol. Chem. 289, 19556–19569. doi:10.1074/jbc.M114.567925

Chen, X., Winters, C., Azzam, R., Li, X., Galbraith, J.A., Leapman, R.D., Reese, T.S., 2008. Organization of the core structure of the postsynaptic density. Proc. Natl. Acad. Sci. U.S.A. 105, 4453–4458. doi:10.1073/pnas.0800897105

Cheng, Z., Teo, G., Krueger, S., Rock, T.M., Koh, H.W.L., Choi, H., Vogel, C., 2016. Differential dynamics of the mammalian mRNA and protein expression response to misfolding stress. Molecular Systems Biology 12, 855.

Deisseroth, K., Bito, H., Tsien, R.W., 1996. Signaling from synapse to nucleus: postsynaptic CREB phosphorylation during multiple forms of hippocampal synaptic plasticity. Neuron 16, 89–101.

Diana, G., Valentini, G., Travaglione, S., Falzano, L., Pieri, M., Zona, C., Meschini, S., Fabbri, A., Fiorentini, C., 2007. Enhancement of learning and memory after activation of cerebral Rho GTPases. Proc. Natl. Acad. Sci. U.S.A. 104, 636–641. doi:10.1073/pnas.0610059104

Dingledine, R., Borges, K., Bowie, D., Traynelis, S.F., 1999. The glutamate receptor ion channels. Pharmacol. Rev. 51, 7–61.

Domon, B., Aebersold, R., 2010. Options and considerations when selecting a quantitative proteomics strategy. Nat. Biotechnol. 28, 710–721. doi:10.1038/nbt.1661

Emes, R.D., Pocklington, A.J., Anderson, C.N.G., Bayes, A., Collins, M.O., Vickers, C.A., Croning, M.D.R., Malik, B.R., Choudhary, J.S., Armstrong, J.D., Grant, S.G.N., 2008. Evolutionary expansion and anatomical specialization of synapse proteome complexity. Nat. Neurosci. 11, 799–806. doi:10.1038/nn.2135

Exton-McGuinness, M.T.J., Patton, R.C., Sacco, L.B., Lee, J.L.C., 2014. Reconsolidation of a well-learned instrumental memory. Learn. Mem. 21, 468–477. doi:10.1101/lm.035543.114

Filiou, M.D., Martins-de-Souza, D., Guest, P.C., Bahn, S., Turck, C.W., 2012. To label or not to label: applications of quantitative proteomics in neuroscience research. Proteomics 12, 736–747.

doi:10.1002/pmic.201100350

Fioravante, D., Byrne, J.H., 2011. Protein degradation and memory formation. Brain Res. Bull. 85, 14–20. doi:10.1016/j.brainresbull.2010.11.002

Frey, M.C., Sprengel, R., Nevian, T., 2009. Activity pattern-dependent long-term potentiation in neocortex and hippocampus of GluA1 (GluR-A) subunit-deficient mice. J. Neurosci. 29, 5587–5596. doi:10.1523/JNEUROSCI.5314-08.2009

Geiger, T., Wehner, A., Schaab, C., Cox, J., Mann, M., 2012. Comparative proteomic analysis of eleven common cell lines reveals ubiquitous but varying expression of most proteins. Mol. Cell Proteomics 11, M111.014050. doi:10.1074/mcp.M111.014050

Geiger, T., Wisniewski, J.R., Cox, J., Zanivan, S., Kruger, M., Ishihama, Y., Mann, M., 2011. Use of stable isotope labeling by amino acids in cell culture as a spike-in standard in quantitative proteomics. Nat Protoc 6, 147–157. doi:10.1038/nprot.2010.192

Giovannini, M.G., Blitzer, R.D., Wong, T., Asoma, K., Tsokas, P., Morrison, J.H., Iyengar, R., Landau, E.M., 2001. Mitogen-activated protein kinase regulates early phosphorylation and delayed expression of Ca2+/calmodulin-dependent protein kinase II in long-term potentiation. J. Neurosci. 21, 7053–7062.

Granger, A.J., Shi, Y., Lu, W., Cerpas, M., Nicoll, R.A., 2013. LTP requires a reserve pool of glutamate receptors independent of subunit type. Nature 493, 495–500. doi:10.1038/nature11775

Gygi, S.P., Rist, B., Gerber, S.A., Turecek, F., Gelb, M.H., Aebersold, R., 1999. Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. Nat. Biotechnol. 17, 994–999. doi:10.1038/13690

Hayashi, Y., Shi, S.H., Esteban, J.A., Piccini, A., Poncer, J.C., Malinow, R., 2000. Driving AMPA receptors into synapses by LTP and CaMKII: requirement for GluR1 and PDZ domain interaction. Science 287, 2262–2267.

Hebert, A.S., Merrill, A.E., Bailey, D.J., Still, A.J., Westphall, M.S., Strieter, E.R., Pagliarini, D.J., Coon, J.J., 2013. Neutron-encoded mass signatures for multiplexed proteome quantification. Nat. Methods 10, 332–334. doi:10.1038/nmeth.2378

Hernandez, P.J., Kelley, A.E., 2004. Long-term memory for instrumental responses does not undergo protein synthesis-dependent reconsolidation upon retrieval. Learning & Memory 11, 748–754. doi:10.1101/lm.84904

Hong, W., 2005. SNAREs and traffic. Biochim. Biophys. Acta 1744, 120–144. doi:10.1016/j.bbamcr.2005.03.014

Hölscher, C., Anwyl, R., Rowan, M.J., 1997. Stimulation on the positive phase of hippocampal theta rhythm induces long-term potentiation that can Be depotentiated by stimulation on the negative phase in area CA1 in vivo. Journal of Neuroscience 17, 6470–6477.

Huang, C.-C., Yang, D.-M., Lin, C.-C., Kao, L.-S., 2011. Involvement of Rab3A in vesicle priming during exocytosis: interaction with Munc13-1 and Munc18-1. Traffic 12, 1356–1370.

doi:10.1111/j.1600-0854.2011.01237.x

Huang, D.W., Sherman, B.T., Tan, Q., Kir, J., Liu, D., Bryant, D., Guo, Y., Stephens, R., Baseler, M.W., Lane, H.C., Lempicki, R.A., 2007. DAVID Bioinformatics Resources: expanded annotation database and novel algorithms to better extract biology from large gene lists. Nucleic Acids Research 35, W169–75. doi:10.1093/nar/gkm415

Izquierdo, I., Bevilaqua, L.R.M., Rossato, J.I., Bonini, J.S., Medina, J.H., Cammarota, M., 2006. Different molecular cascades in different sites of the brain control memory consolidation. Trends Neurosci. 29, 496–505. doi:10.1016/j.tins.2006.07.005

Jackson, R.J., Hellen, C.U.T., Pestova, T.V., 2010. The mechanism of eukaryotic translation initiation and principles of its regulation. Nat. Rev. Mol. Cell Biol. 11, 113–127. doi:10.1038/nrm2838

Jarome, T.J., Ferrara, N.C., Kwapis, J.L., Helmstetter, F.J., 2016. CaMKII regulates proteasome phosphorylation and activity and promotes memory destabilization following retrieval. Neurobiol Learn Mem 128, 103–109. doi:10.1016/j.nlm.2016.01.001

Jurado-Parras, M.T., Sánchez-Campusano, R., Castellanos, N.P., del-Pozo, F., Gruart, A., Delgado-García, J.M., 2013. Differential contribution of hippocampal circuits to appetitive and consummatory behaviors during operant conditioning of behaving mice. J. Neurosci. 33, 2293–2304. doi:10.1523/JNEUROSCI.1013-12.2013

Kaltschmidt, B., Ndiaye, D., Korte, M., Pothion, S., Arbibe, L., Prüllage, M., Pfeiffer, J., Lindecke, A., Staiger, V., Israël, A., Kaltschmidt, C., Mémet, S., 2006. NF-kappaB regulates spatial memory formation and synaptic plasticity through protein kinase A/CREB signaling. Mol. Cell. Biol. 26, 2936–2946. doi:10.1128/MCB.26.8.2936-2946.2006

Kandel, E.R., Dudai, Y., Mayford, M.R., 2014. The molecular and systems biology of memory. Cell 157, 163–186. doi:10.1016/j.cell.2014.03.001

Karp, N.A., Huber, W., Sadowski, P.G., Charles, P.D., Hester, S.V., Lilley, K.S., 2010. Addressing accuracy and precision issues in iTRAQ quantitation. Mol. Cell Proteomics 9, 1885–1897. doi:10.1074/mcp.M900628-MCP200

Kent, C.B., Shimada, T., Ferraro, G.B., Ritter, B., Yam, P.T., McPherson, P.S., Charron, F., Kennedy, T.E., Fournier, A.E., 2010. 14-3-3 proteins regulate protein kinase a activity to modulate growth cone turning responses. J. Neurosci. 30, 14059–14067. doi:10.1523/JNEUROSCI.3883-10.2010

Kettenbach, A.N., Rush, J., Gerber, S.A., 2011. Absolute quantification of protein and post-translational modification abundance with stable isotope-labeled synthetic peptides. Nat Protoc 6, 175–186. doi:10.1038/nprot.2010.196

Klann, E., Dever, T.E., 2004. Biochemical mechanisms for translational regulation in synaptic plasticity. Nat. Rev. Neurosci. 5, 931–942. doi:10.1038/nrn1557

Kruger, M., Moser, M., Ussar, S., Thievessen, I., Luber, C.A., Forner, F., Schmidt, S., Zanivan, S., Fässler, R., Mann, M., 2008. SILAC mouse for quantitative proteomics uncovers kindlin-3 as

an essential factor for red blood cell function. Cell 134, 353–364. doi:10.1016/j.cell.2008.05.033

Lamprecht, R., 2011. The roles of the actin cytoskeleton in fear memory formation. Front Behav Neurosci 5, 39. doi:10.3389/fnbeh.2011.00039

Lee, J.L.C., Nader, K., Schiller, D., 2017. An Update on Memory Reconsolidation Updating. Trends Cogn. Sci. (Regul. Ed.) 21, 531–545. doi:10.1016/j.tics.2017.04.006

Lee, S.-H., Choi, J.-H., Lee, N., Lee, H.-R., Kim, J.-I., Yu, N.-K., Choi, S.-L., Lee, S.-H., Kim, H., Kaang, B.-K., 2008. Synaptic protein degradation underlies destabilization of retrieved fear memory. Science 319, 1253–1256. doi:10.1126/science.1150541

Lesburguères, E., Gobbo, O.L., Alaux-Cantin, S., Hambucken, A., Trifilieff, P., Bontempi, B., 2011. Early tagging of cortical networks is required for the formation of enduring associative memory. Science 331, 924–928. doi:10.1126/science.1196164

Lisman, J., Schulman, H., Cline, H., 2002. The molecular basis of CaMKII function in synaptic and behavioural memory. Nat. Rev. Neurosci. 3, 175–190. doi:10.1038/nrn753

Lisman, J., Yasuda, R., Raghavachari, S., 2012. Mechanisms of CaMKII action in long-term potentiation. Nat. Rev. Neurosci. 13, 169–182. doi:10.1038/nrn3192

Liu, Y., Beyer, A., Aebersold, R., 2016. On the Dependency of Cellular Protein Levels on mRNA Abundance. Cell 165, 535–550. doi:10.1016/j.cell.2016.03.014

Lorenzetti, F.D., Baxter, D.A., Byrne, J.H., 2008. Molecular mechanisms underlying a cellular analog of operant reward learning. Neuron 59, 815–828. doi:10.1016/j.neuron.2008.07.019

Lu, R., Markowetz, F., Unwin, R.D., Leek, J.T., Airoldi, E.M., MacArthur, B.D., Lachmann, A., Rozov, R., Ma'ayan, A., Boyer, L.A., Troyanskaya, O.G., Whetton, A.D., Lemischka, I.R., 2009. Systems-level dynamic analyses of fate change in murine embryonic stem cells. Nature 462, 358–362. doi:10.1038/nature08575

Lu, W., Isozaki, K., Roche, K.W., Nicoll, R.A., 2010. Synaptic targeting of AMPA receptors is regulated by a CaMKII site in the first intracellular loop of GluA1. Proc. Natl. Acad. Sci. U.S.A. 107, 22266–22271. doi:10.1073/pnas.1016289107

Lüscher, C., Malenka, R.C., 2012. NMDA receptor-dependent long-term potentiation and long-term depression (LTP/LTD). Cold Spring Harb Perspect Biol 4. doi:10.1101/cshperspect.a005710

Ma, T., Chen, Y., Vingtdeux, V., Zhao, H., Viollet, B., Marambaud, P., Klann, E., 2014. Inhibition of AMP-activated protein kinase signaling alleviates impairments in hippocampal synaptic plasticity induced by amyloid β. J. Neurosci. 34, 12230–12238. doi:10.1523/JNEUROSCI.1694-14.2014

Mann, M., Kelleher, N.L., 2008. Precision proteomics: the case for high resolution and high mass accuracy. Proc. Natl. Acad. Sci. U.S.A. 105, 18132–18138. doi:10.1073/pnas.0800788105

Mann, M., Kulak, N.A., Nagaraj, N., Cox, J., 2013. The coming age of complete, accurate, and ubiquitous proteomes. Mol. Cell 49, 583–590. doi:10.1016/j.molcel.2013.01.029

Marshall, A.G., Hendrickson, C.L., Jackson, G.S., 1998. Fourier transform ion cyclotron resonance mass spectrometry: a primer. Mass Spectrom Rev 17, 1–35. doi:10.1002/(SICI)1098-2787(1998)17:1<1::AID-MAS1>3.0.CO;2-K

Martineau, Y., Wang, X., Alain, T., Petroulakis, E., Shahbazian, D., Fabre, B., Bousquet-Dubouch, M.-P., Monsarrat, B., Pyronnet, S., Sonenberg, N., 2014. Control of Paip1-eukayrotic translation initiation factor 3 interaction by amino acids through S6 kinase. Mol. Cell. Biol. 34, 1046–1053. doi:10.1128/MCB.01079-13

McGaugh, J.L., 2000. Memory--a century of consolidation. Science 287, 248–251.

McMahon, H.T., Missler, M., Li, C., Südhof, T.C., 1995. Complexins: cytosolic proteins that regulate SNAP receptor function. Cell 83, 111–119.

Meissner, F., Mann, M., 2014. Quantitative shotgun proteomics: considerations for a high-quality workflow in immunology. Nat. Immunol. 15, 112–117. doi:10.1038/ni.2781

Michalski, A., Damoc, E., Lange, O., Denisov, E., Nolting, D., Müller, M., Viner, R., Schwartz, J., Remes, P., Belford, M., Dunyach, J.-J., Cox, J., Horning, S., Mann, M., Makarov, A., 2012. Ultra high resolution linear ion trap Orbitrap mass spectrometer (Orbitrap Elite) facilitates top down LC MS/MS and versatile peptide fragmentation modes. Mol. Cell Proteomics 11, O111.013698. doi:10.1074/mcp.O111.013698

Milekic, M.H., Alberini, C.M., 2002. Temporally graded requirement for protein synthesis following memory reactivation. Neuron 36, 521–525.

Monopoli, M.P., Raghnaill, M.N., Loscher, J.S., O'Sullivan, N.C., Pangalos, M.N., Ring, R.H., Schack, von, D., Dunn, M.J., Regan, C.M., Pennington, S., Murphy, K.J., 2011. Temporal proteomic profile of memory consolidation in the rat hippocampal dentate gyrus. Proteomics 11, 4189–4201. doi:10.1002/pmic.201100072

Moser, M.-B., Rowland, D.C., Moser, E.I., 2015. Place cells, grid cells, and memory. Cold Spring Harb Perspect Biol 7, a021808. doi:10.1101/cshperspect.a021808

Mueller, S., Wahlander, A., Selevsek, N., Otto, C., Ngwa, E.M., Poljak, K., Frey, A.D., Aebi, M., Gauss, R., 2015. Protein degradation corrects for imbalanced subunit stoichiometry in OST complex assembly. Mol. Biol. Cell 26, 2596–2608. doi:10.1091/mbc.E15-03-0168

Musunuri, S., Wetterhall, M., Ingelsson, M., Lannfelt, L., Artemenko, K., Bergquist, J., Kultima, K., Shevchenko, G., 2014. Quantification of the brain proteome in Alzheimer's disease using multiplexed mass spectrometry. J. Proteome Res. 13, 2056–2068. doi:10.1021/pr401202d

Nabavi, S., Fox, R., Proulx, C.D., Lin, J.Y., Tsien, R.Y., Malinow, R., 2014. Engineering a memory with LTD and LTP. Nature 511, 348–352. doi:10.1038/nature13294

Nagaraj, N., Wisniewski, J.R., Geiger, T., Cox, J., Kircher, M., Kelso, J., Pääbo, S., Mann, M., 2011. Deep proteome and transcriptome mapping of a human cancer cell line. Molecular Systems Biology 7, 548–548. doi:10.1038/msb.2011.81

Nepusz, T., Yu, H., Paccanaro, A., 2012. Detecting overlapping protein complexes in protein-protein interaction networks. Nat. Methods 9, 471–472. doi:10.1038/nmeth.1938

Neves, G., Cooke, S.F., Bliss, T.V.P., 2008. Synaptic plasticity, memory and the hippocampus: a neural network approach to causality. Nat. Rev. Neurosci. 9, 65–75. doi:10.1038/nrn2303

Nguyen, P.V., Abel, T., Kandel, E.R., 1994. Requirement of a critical period of transcription for induction of a late phase of LTP. Science 265, 1104–1107.

Ong, S.-E., Blagoev, B., Kratchmarova, I., Kristensen, D.B., Steen, H., Pandey, A., Mann, M., 2002. Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. Mol. Cell Proteomics 1, 376–386.

Ong, S.-E., Mann, M., 2005. Mass spectrometry-based proteomics turns quantitative. Nat. Chem. Biol. 1, 252–262. doi:10.1038/nchembio736

Park, M., Salgado, J.M., Ostroff, L., Helton, T.D., Robinson, C.G., Harris, K.M., Ehlers, M.D., 2006. Plasticity-induced growth of dendritic spines by exocytic trafficking from recycling endosomes. Neuron 52, 817–830. doi:10.1016/j.neuron.2006.09.040

Patterson, S.L., Pittenger, C., Morozov, A., Martin, K.C., Scanlin, H., Drake, C., Kandel, E.R., 2001. Some forms of cAMP-mediated long-lasting potentiation are associated with release of BDNF and nuclear translocation of phospho-MAP kinase. Neuron 32, 123–140.

Pontes, A.H., de Sousa, M.V., 2016. Mass Spectrometry-Based Approaches to Understand the Molecular Basis of Memory. Front Chem 4, 40. doi:10.3389/fchem.2016.00040

Poser, S., Storm, D.R., 2001. Role of Ca2+-stimulated adenylyl cyclases in LTP and memory formation. International Journal of Developmental Neuroscience 19, 387–394. doi:10.1016/S0736-5748(00)00094-0

Qiao, H., Foote, M., Graham, K., Wu, Y., Zhou, Y., 2014. 14-3-3 proteins are required for hippocampal long-term potentiation and associative learning and memory. J. Neurosci. 34, 4801–4808. doi:10.1523/JNEUROSCI.4393-13.2014

Rao-Ruiz, P., Carney, K.E., Pandya, N., van der Loo, R.J., Verheijen, M.H.G., van Nierop, P., Smit, A.B., Spijker, S., 2015. Time-dependent changes in the mouse hippocampal synaptic membrane proteome after contextual fear conditioning. Hippocampus 25, 1250–1261. doi:10.1002/hipo.22432

Rapanelli, M., Frick, L.R., Zanutto, B.S., 2011. Learning an operant conditioning task differentially induces gliogenesis in the medial prefrontal cortex and neurogenesis in the hippocampus. PLoS ONE 6, e14713. doi:10.1371/journal.pone.0014713

Rappsilber, J., Mann, M., Ishihama, Y., 2007. Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips. Nat Protoc 2, 1896–1906. doi:10.1038/nprot.2007.261

Rauniyar, N., McClatchy, D.B., Yates, J.R., 2013. Stable isotope labeling of mammals (SILAM) for in vivo quantitative proteomic analysis. Methods 61, 260–268. doi:10.1016/j.ymeth.2013.03.008

Rempel-Clower, N.L., Zola, S.M., Squire, L.R., Amaral, D.G., 1996. Three cases of enduring memory impairment after bilateral damage limited to the hippocampal formation. Journal of

Neuroscience 16, 5233–5255.
Roberson, E.D., English, J.D., Adams, J.P., Selcher, J.C., Kondratick, C., Sweatt, J.D., 1999. The Mitogen-Activated Protein Kinase Cascade Couples PKA and PKC to cAMP Response Element Binding Protein Phosphorylation in Area CA1 of Hippocampus. Journal of Neuroscience 19, 4337–4348. doi:10.1523/JNEUROSCI.19-11-04337.1999
Ross, C.A., Poirier, M.A., 2004. Protein aggregation and neurodegenerative disease. Nat. Med. 10 Suppl, S10–7. doi:10.1038/nm1066
Ross, P.L., Huang, Y.N., Marchese, J.N., Williamson, B., Parker, K., Hattan, S., Khainovski, N., Pillai, S., Dey, S., Daniels, S., Purkayastha, S., Juhasz, P., Martin, S., Bartlet-Jones, M., He, F., Jacobson, A., Pappin, D.J., 2004. Multiplexed protein quantitation in Saccharomyces cerevisiae using amine-reactive isobaric tagging reagents. Mol. Cell Proteomics 3, 1154–1169. doi:10.1074/mcp.M400129-MCP200
Routtenberg, A., Rekart, J.L., 2005. Post-translational protein modification as the substrate for long-lasting memory. Trends Neurosci. 28, 12–19. doi:10.1016/j.tins.2004.11.006
Ryan, M.M., Ryan, B., Kyrke-Smith, M., Logan, B., Tate, W.P., Abraham, W.C., Williams, J.M., 2012. Temporal profiling of gene networks associated with the late phase of long-term potentiation in vivo. PLoS ONE 7, e40538. doi:10.1371/journal.pone.0040538
Ryan, T.J., Grant, S.G.N., 2009. The origin and evolution of synapses. Nat. Rev. Neurosci. 10, 701–712. doi:10.1038/nrn2717
Ryan, T.J., Roy, D.S., Pignatelli, M., Arons, A., Tonegawa, S., 2015. Memory. Engram cells retain memory under retrograde amnesia. Science 348, 1007–1013. doi:10.1126/science.aaa5542
Sadygov, R.G., Cociorva, D., Yates, J.R., 2004. Large-scale database searching using tandem mass spectra: looking up the answer in the back of the book. Nat. Methods 1, 195–202. doi:10.1038/nmeth725
Schiller, J., Schiller, Y., Clapham, D.E., 1998. NMDA receptors amplify calcium influx into dendritic spines during associative pre- and postsynaptic activation. Nat. Neurosci. 1, 114–118. doi:10.1038/363
Schwämmle, V., Jensen, O.N., 2010. A simple and fast method to determine the parameters for fuzzy c-means cluster analysis. Bioinformatics 26, 2841–2848. doi:10.1093/bioinformatics/btq534
Schwämmle, V., León, I.R., Jensen, O.N., 2013. Assessment and improvement of statistical tools for comparative proteomics analysis of sparse data sets with few experimental replicates. J. Proteome Res. 12, 3874–3883. doi:10.1021/pr400045u
Sharma, K., Schmitt, S., Bergner, C.G., Tyanova, S., Kannaiyan, N., Manrique-Hoyos, N., Kongi, K., Cantuti, L., Hanisch, U.-K., Philips, M.-A., Rossner, M.J., Mann, M., Simons, M., 2015. Cell type- and brain region-resolved mouse brain proteome. Nat. Neurosci. 18, 1819–1831. doi:10.1038/nn.4160
Skoulakis, E.M., Davis, R.L., 1996. Olfactory learning deficits in mutants for leonardo, a Drosophila

gene encoding a 14-3-3 protein. Neuron 17, 931–944.
Squire, L.R., Genzel, L., Wixted, J.T., Morris, R.G., 2015. Memory consolidation. Cold Spring Harb Perspect Biol 7, a021766. doi:10.1101/cshperspect.a021766
Strange, B.A., Witter, M.P., Lein, E.S., Moser, E.I., 2014. Functional organization of the hippocampal longitudinal axis. Nat. Rev. Neurosci. 15, 655–669. doi:10.1038/nrn3785
Strekalova, T., Zörner, B., Zacher, C., Sadovska, G., Herdegen, T., Gass, P., 2003. Memory retrieval after contextual fear conditioning induces c-Fos and JunB expression in CA1 hippocampus. Genes Brain Behav. 2, 3–10.
Sui, L., Wang, J., Li, B.-M., 2008. Role of the phosphoinositide 3-kinase-Akt-mammalian target of the rapamycin signaling pathway in long-term potentiation and trace fear conditioning memory in rat medial prefrontal cortex. Learn. Mem. 15, 762–776. doi:10.1101/lm.1067808
Szklarczyk, D., Morris, J.H., Cook, H., Kuhn, M., Wyder, S., Simonovic, M., Santos, A., Doncheva, N.T., Roth, A., Bork, P., Jensen, L.J., Mering, von, C., 2017. The STRING database in 2017: quality-controlled protein-protein association networks, made broadly accessible. Nucleic Acids Research 45, D362–D368. doi:10.1093/nar/gkw937
Thakur, S.S., Geiger, T., Chatterjee, B., Bandilla, P., Fröhlich, F., Cox, J., Mann, M., 2011. Deep and highly sensitive proteome coverage by LC-MS/MS without prefractionation. Mol. Cell Proteomics 10, M110.003699. doi:10.1074/mcp.M110.003699
Thingholm, T.E., Jensen, O.N., Robinson, P.J., Larsen, M.R., 2008. SIMAC (sequential elution from IMAC), a phosphoproteomics strategy for the rapid separation of monophosphorylated from multiply phosphorylated peptides. Mol. Cell Proteomics 7, 661–671. doi:10.1074/mcp.M700362-MCP200
Thompson, A., Schäfer, J., Kuhn, K., Kienle, S., Schwarz, J., Schmidt, G., Neumann, T., Johnstone, R., Mohammed, A.K.A., Hamon, C., 2003. Tandem mass tags: a novel quantification strategy for comparative analysis of complex protein mixtures by MS/MS. Anal. Chem. 75, 1895–1904.
Ting, L., Rad, R., Gygi, S.P., Haas, W., 2011. MS3 eliminates ratio distortion in isobaric multiplexed quantitative proteomics. Nat. Methods 8, 937–940. doi:10.1038/nmeth.1714
Tronson, N.C., Taylor, J.R., 2007. Molecular mechanisms of memory reconsolidation. Nat. Rev. Neurosci. 8, 262–275. doi:10.1038/nrn2090
Tsokas, P., Hsieh, C., Yao, Y., Lesburguères, E., Wallace, E.J.C., Tcherepanov, A., Jothianandan, D., Hartley, B.R., Pan, L., Rivard, B., Farese, R.V., Sajan, M.P., Bergold, P.J., Hernández, A.I., Cottrell, J.E., Shouval, H.Z., Fenton, A.A., Sacktor, T.C., 2016. Compensation for PKMζ in long-term potentiation and spatial long-term memory in mutant mice. Elife 5, 12677. doi:10.7554/eLife.14846
Tucker, W.C., Chapman, E.R., 2002. Role of synaptotagmin in Ca2+-triggered exocytosis. Biochem. J. 366, 1–13. doi:10.1042/BJ20020776
Uzakov, S., Frey, J.U., Korz, V., 2005. Reinforcement of rat hippocampal LTP by holeboard training. Learning & Memory 12, 165–171. doi:10.1101/lm.89305

Villén, J., Beausoleil, S.A., Gerber, S.A., Gygi, S.P., 2007. Large-scale phosphorylation analysis of mouse liver. Proc. Natl. Acad. Sci. U.S.A. 104, 1488–1493. doi:10.1073/pnas.0609836104

Viola, H., Furman, M., Izquierdo, L.A., Alonso, M., Barros, D.M., de Souza, M.M., Izquierdo, I., Medina, J.H., 2000. Phosphorylated cAMP response element-binding protein as a molecular marker of memory processing in rat hippocampus: effect of novelty. J. Neurosci. 20, RC112.

Vizcaíno, J.A., Csordas, A., del-Toro, N., Dianes, J.A., Griss, J., Lavidas, I., Mayer, G., Perez-Riverol, Y., Reisinger, F., Ternent, T., Xu, Q.-W., Wang, R., Hermjakob, H., 2016. 2016 update of the PRIDE database and its related tools. Nucleic Acids Research 44, D447–56. doi:10.1093/nar/gkv1145

Wang, H., Yang, Y., Li, Y., Bai, B., Wang, X., Tan, H., Liu, T., Beach, T.G., Peng, J., Wu, Z., 2015. Systematic optimization of long gradient chromatography mass spectrometry for deep analysis of brain proteome. J. Proteome Res. 14, 829–838. doi:10.1021/pr500882h

Wang, X., Pattison, J.S., Su, H., 2013. Posttranslational modification and quality control. Circ. Res. 112, 367–381. doi:10.1161/CIRCRESAHA.112.268706

Wenger, C.D., Lee, M.V., Hebert, A.S., McAlister, G.C., Phanstiel, D.H., Westphall, M.S., Coon, J.J., 2011. Gas-phase purification enables accurate, multiplexed proteome quantification with isobaric tagging. Nat. Methods 8, 933–935. doi:10.1038/nmeth.1716

Whitlock, J.R., Heynen, A.J., Shuler, M.G., Bear, M.F., 2006. Learning induces long-term potentiation in the hippocampus. Science 313, 1093–1097. doi:10.1126/science.1128134

Wisniewski, J.R., Zougman, A., Mann, M., 2009. Combination of FASP and StageTip-based fractionation allows in-depth analysis of the hippocampal membrane proteome. J. Proteome Res. 8, 5674–5678. doi:10.1021/pr900748n

Wong, S.T., Athos, J., Figueroa, X.A., Pineda, V.V., Schaefer, M.L., Chavkin, C.C., Muglia, L.J., Storm, D.R., 1999. Calcium-stimulated adenylyl cyclase activity is critical for hippocampus-dependent long-term memory and late phase LTP. Neuron 23, 787–798.

Yi, E.C., Li, X.-J., Cooke, K., Lee, H., Raught, B., Page, A., Aneliunas, V., Hieter, P., Goodlett, D.R., Aebersold, R., 2005. Increased quantitative proteome coverage with (13)C/(12)C-based, acid-cleavable isotope-coded affinity tag reagent and modified data acquisition scheme. Proteomics 5, 380–387. doi:10.1002/pmic.200400970

Zhang, Y., Fonslow, B.R., Shan, B., Baek, M.-C., Yates, J.R., 2013. Protein analysis by shotgun/bottom-up proteomics. Chem. Rev. 113, 2343–2394. doi:10.1021/cr3003533

Zhou, M., Sandercock, A.M., Fraser, C.S., Ridlova, G., Stephens, E., Schenauer, M.R., Yokoi-Fong, T., Barsky, D., Leary, J.A., Hershey, J.W., Doudna, J.A., Robinson, C.V., 2008. Mass spectrometry reveals modularity and a complete subunit interaction map of the eukaryotic translation factor eIF3. Proc. Natl. Acad. Sci. U.S.A. 105, 18139–18144. doi:10.1073/pnas.0801313105

Zola-Morgan, S., Squire, L.R., Amaral, D.G., 1986. Human amnesia and the medial temporal region: enduring memory impairment following a bilateral lesion limited to field CA1 of the

hippocampus. Journal of Neuroscience 6, 2950–2967.

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