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Quantitative proteomics of auditory fear conditioning

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ABSTRACT

Auditory fear conditioning is a well-characterized rodent learning model where a neutral auditory cue is paired with an aversive outcome to induce associative fear memory. The storage of long-term auditory fear memory requires long-term potentiation (LTP) in the lateral amygdala and *de novo* protein synthesis. Although many studies focused on individual proteins have shown their contribution to LTP and fear conditioning, non-biased genome-wide studies have only recently been possible with microarrays, which nevertheless fall short of measuring changes at the level of proteins. Here we employed quantitative proteomics to examine the expression of hundreds of proteins in the lateral amygdala in response to auditory fear conditioning. We found that various proteins previously implicated in LTP, learning and axon/dendrite growth were regulated by fear conditioning. A substantial number of proteins that were regulated by fear conditioning have not yet been studied specifically in learning or synaptic plasticity.

1. Introduction

Learning and memory involve intricate molecular mechanisms in the brain at the transcriptional, translational, and post-translational level [1]. Whereas short-term memory does not require translation, long-term memory persists for hours to years and is known to require *de novo* protein synthesis [2]. This protein-synthesis dependent stabilization of memory into an enduring engram is called cellular consolidation [3]. A particularly well characterized learning paradigm is auditory-cued fear conditioning, which largely depends on the lateral amygdala (LA) [4–7]. Lateral amygdala input synapses are known to undergo long-term potentiation (LTP) upon fear conditioning [8,9], and this synaptic potentiation is crucial for learning the association between a neutral cue and an aversive outcome [10–12].

Which proteins are important in supporting the long-term memory engram? Although many studies focused on individual proteins have shown their contribution to synaptic plasticity and memory, non-biased genome-wide studies have only begun with the development of microarrays. Fear conditioning has also been studied through microarrays [13–15], but while microarray data provide a genome-wide profile of gene expression, it still falls short of measuring translational and post-translational changes.

Mass spectrometry-based proteomics has single amino-acid sequence level resolution and offers an unprecedented detail in the biochemical assessment of biological processes. The breakthroughs in quantitative proteomics have largely accelerated research on proteome-wide network changes and the identification of previously unknown molecules [16–18]. Isobaric tags such as TMT and iTRAQ have enabled the comparison of expression levels of thousands of proteins across complex samples.

Here we use TMT tagging to explore proteome-wide changes upon associative learning. We employ the rat auditory fear conditioning model and compare protein expression in the lateral amygdala of naïve and fear conditioned animals. Unpaired controls, which received auditory cues and aversive footshock identical to the conditioned rats in an incongruous manner to prevent association, were used to discern changes that were specific to associative learning. Our results represent a quantitative proteomic approach to reveal the underlying translational mechanisms of auditory fear memory.

2. Materials and methods

2.1. Experimental animals

Male Sprague–Dawley rats (3–5 weeks old) were obtained from Orient Bio Inc. (Seongnam, Republic of Korea). Rats were housed in

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plastic cages and fed *ad libitum* under an inverted 12/12 light/dark cycle (light off at 9:00 AM). Behavioral training was done during the dark portion of the light/dark cycle. All behavioral procedures were approved by Institute of Laboratory Animal Resources of Seoul National University. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2. Fear conditioning

For fear conditioning, rats were placed in a Plexiglas conditioning chamber and left undisturbed for 2 min [19–22]. A neutral tone (CS; 30 s, 2.8 kHz, 85 dB) co-terminating with an electrical foot shock (US; 1.0 mA, 1 s) was then presented three times with an average interval of 100 s. Rats were returned to their home cages 60 s after the last shock was applied. Twenty-four hours after conditioning, a subgroup of rats was presented with a tone (30 s, 2.8 kHz, 85 dB) in a distinct context to test memory retention. A separate subgroup of animals (18 in total: 6 rats per group without pooling) was used for proteomic analysis.

2.3. Slice preparation and sample preparation

Rats were anesthetized with isoflurane and decapitated to remove the brain as previously described [19]. The isolated whole brains were placed in an ice-cold modified aCSF solution containing (in mM) 175 sucrose, 20 NaCl, 3.5 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 1.3 $MgCl_2$ and 11 D-(+)-glucose. Solutions were gassed with 95% O_2/5% CO_2. Coronal slices (400 μm) including the lateral amygdala were cut using a vibroslicer (NVSL, World Precision Instruments, Sarasota, FL) and the lateral amygdala was dissected and frozen immediately. Lateral amygdala tissue samples were homogenized in ice-cold modified RIPA buffer containing 50 mM Tris (pH 7.6), 150 mM NaCl, 5 mM NaF, 1 mM Na_3VO_4, 0.5% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS and protease/phosphatase inhibitor cocktail. Samples were sonicated, boiled for 10 min, and spun down at 15,000*g* at 4 °C for 15 min. Protein content was quantified by a bicinchoninic acid assay (Thermo Scientific) following the manufacturer's protocol, and confirmed by SDS–PAGE and silver staining.

2.4. TMT labeling

Samples were subsequently tagged with tandem mass tags for quantitative mass spectrometry (TMTsixplex™ Isobaric Mass Tagging Kit, Thermo Scientific). Briefly, 100 μg of protein was taken from each sample and was reduced with 500 mM tris(2-carboxyethyl)phosphine (TCEP) at 55 °C for 1 h and then alkylated with 300 mM iodoacetamide (IAA) at 37 °C in the dark for 30 min. The samples were desalted using a 10,000 MW-cutoff membrane filter and dissolved in 100 mM triethylammonium bicarbonate (TEAB) buffer to a final concentration of 1 μg/μl. Sequencing grade trypsin (Promega, Madison, WI, USA) was added at 1:100 (w/w) into proteins in TEAB buffer and incubated overnight at 37 °C. The eighteen samples (six samples per group) were individually labeled using TMT-126, 129 (naïve controls), TMT-127, 130 (unpaired controls) and TMT-128, 131 (fear conditioned group) following the manufacturer's instructions. Aqueous hydroxylamine solution (5% w/v) was added to quench the reaction. Two samples from the three groups (6 samples) were then combined into 6-plex solutions for comparison, speed-vacuum dried, and then dissolved in 50 μl of water containing 0.1% formic acid for LC-MS/MS analysis. Finally, three batches of TMT-6-plex labeled peptides were prepared to compare a total of six samples per group ($n = 6$).

2.5. 2D-LC-MS/MS

The TMT-labeled samples were analyzed using a 2D-LC-MS/MS system consisting of a nanoACQUITY UltraPerformance LC System (Waters, USA) and a LTQ Orbitrap Elite mass spectrometer (Thermo Scientific, USA) equipped with a nano-electrospray source. A detailed description of 2D-LC-MS/MS analysis can be found in previous literatures [23,24]. Briefly, a strong cation exchange (5 μm, 3 cm) column was placed just before the C_{18} trap column (id 180 μm, length 20 mm, and particle size 5 μm; Waters). Peptide solutions were loaded in 5 μl aliquots for each run. Peptides were displaced from the strong cation exchange phase to the C_{18} phase by a salt gradient that was introduced through an autosampler loop and then desalted for 10 min at a flow rate of 4 μl/min. Then, the trapped peptides were separated on a 200 mm homemade microcapillary column consisting of C_{18} (Aqua; particle size 3 μm) packed into 100 μm silica tubing with an orifice id of 5 μm.

An eleven-step salt gradient was performed using 3 μl of 0, 25, 50, 100, 250, and 500 mM ammonium acetate (0.1% formic acid in water) and 4, 5, 9 and an additional 9 μl at 500 mM ammonium acetate (0.1% formic acid in 30% ammonium acetate). The mobile phases, A and B, were composed of 0% and 100% acetonitrile, respectively, and each contained 0.1% formic acid. The LC gradient began with 5% B for 1 min and was ramped to 20% B over 5 min, to 35% B over 90 min, to 95% B over 1 min, and remained at 95% B over 13 min and 5% B for another 5 min. The column was re-equilibrated with 5% B for 15 min before the next run. The voltage applied to produce an electrospray was 2.0 kV. During the chromatographic separation, the LTQ Orbitrap Elite was operated in a data-dependent mode. The MS data were acquired using the following parameters: four data-dependent CID-high energy collision dissociation (CID–HCD) dual MS/MS scans per full scan; CID scans were acquired in LTQ with two-microscan averaging; full scans and HCD scans were acquired in Orbitrap at resolution 60,000 and 15,000, respectively, with two-microscan averaging; 35% normalized collision energy in CID and 45% normalized collision energy in HCD; ±1 Da isolation window. Previously fragmented ions were excluded for 60 s. In CID–HCD dual scan, each selected parent ion was first fragmented by CID and then by HCD.

2.6. Protein identification, quantification, and statistical analysis

Probability-based (and error-tolerant) protein database searching of MS/MS spectra against the latest IPI rat protein database (IPI rat v3.70) was performed with a local MASCOT server (2.3, Matrix Science, London, UK) to identify and quantify proteins. The rate of decoy hits in the combined forward and reversed database was less than 1% of the forward hits on both the peptide and the protein levels in each of these experiments. Search criteria were set at: 20 ppm precursor ion mass tolerance, 0.5 Da product ion mass tolerance, two missed cleavages, trypsin as enzyme, TMT modification at the N-terminus and lysine residues and carbamidomethylation at cysteine residues as static modifications, oxidation at methionine, phosphorylation at serine, threonine, and tyrosine as a variable modifications, an ion score threshold of 20 and TMT-6-plex for quantification. Quantification was based on the averaged signal-to-noise ratio of TMT reporter product ions of more than two unique peptides.

Only the 482 proteins identified and quantified across all three TMT experiments were analyzed. The resulting ratios were logarithmized (base = 2) in order to achieve a normal distribution. Median and standard deviation were calculated, and ratio values corrected for the median to account for variability among different pairs [25]. Ratios were averaged and proteins with ratio values beyond $p < 0.05$ in normal distribution were defined as significantly regulated.