Hippocampal Protein Levels Related to Spatial Memory Are Different in the Barnes Maze and in the Multiple T-Maze

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The Multiple T-maze (MTM) and the Barnes maze (BM) are land mazes used for the evaluation of spatial memory. The observation that mice are performing differently in individual mazes made us test the hypothesis that differences in cognitive performances in the two land mazes would be accompanied by differences in hippocampal protein levels. C57BL/6J mice were tested in the BM and in the MTM, hippocampi were extirpated 6 h following the probe trials each, and proteins were extracted for gel-based proteomic analysis. Mice learned the task in both paradigms. Levels of hippocampal proteins from several pathways including signaling, chaperone, and metabolic cascades were significantly different between the two spatial memory tasks. Protein levels were linked to spatial memory specifically as yoked controls were used.

Keywords: Hippocampus • spatial memory • Barnes maze • Multiple T-maze

Introduction

It is well-known and widely accepted that long-term memory is linked and depend on protein expression1–5 and indeed a large series of proteins have been shown to play a role in memory formation using different paradigms or in the proposed electrophysiological correlate of memory, long-term potentiation. Evaluation of spatial memory is carried out by the use of several tasks including the Morris water maze (MWM), or the land mazes Multiple T-maze (MTM) and Barnes maze (BM). Information on protein expression linked to results in these memory tasks is limited, and only recently, memory-related protein expression in the OF1 mouse has been associated with results from the MWM using a systematic proteomic approach by a gel-based proteomic method.6

No data on protein expression in spatial memory tasks of the MTM and BM are available so far. Moreover, different mouse strains show different performance in the individual spatial memory tasks and one and the same mouse strain shows different performance in different spatial memory paradigms.7–10 Different hippocampal protein expression between mouse strains, the absence of systematic studies linking spatial memory in the MTM and BM and the fact that a given mouse strain performs differently in these two mazes formed the Rationale for the current study. It was therefore the aim of this work to use a well-documented and widely used mouse strain, the C57BL/6J,11–13 for linking hippocampal protein expression to spatial memory performance in the MTM and BM using yoked controls to rule out nonspecific protein expression.6 Furthermore, the question of task-specific hippocampal protein expression in both land mazes was addressed.

First evidence for task-related hippocampal levels of proteins from several pathways and cascades was provided herein. This is of relevance for interpretation of studies from the past and the design of future studies, but the fact that different spatial memory paradigms use different protein levels is an intriguing finding per se.

Materials and Methods

Animals. In a previous publication,10 cognitive studies were carried out in the BM and in the MTM. Hippocampal tissue was taken from these animals for proteomics studies carried out herein. Ten to 14-week-old male C57BL/6J mice (19–20 per group, total n = 79) were used for gel-based proteomics studies.

Barnes Maze (BM). With the BM, animals receive reinforcement to escape from the open platform surface to a small dark recessed chamber located under the platform called a “target
hungry for the following day for MTM tests. Mice were trained the amount to preserve their body weight but keeping them with 1% incidin solution. After testing, animals were given food in the cage. Immediately after each trial, the entire maze was cleaned with 1% incidin solution and the maze was rotated to eliminate the use of intramaze cues. Trials were recorded by using computerized tracking/image analyzer system (video camcorder: 1/3 in. SSAMHR EX VIEWHAD coupled with computational tracking system: TiBeSplit). The parameter “time to escape into the tunnel” was recorded and used for correlations with protein data.

Prior to testing, mice were deprived of food for 16 h to motivate food searching. Mice were placed in the middle of the maze in a black colored cylindrical start chamber, and a buzzer (85 dB) was turned on. After 10 s elapsed, the chamber was lifted, and the mouse was pretrained to enter the escape box by guiding it to the escape box and remaining there for 2 min. Following the pretraining trial, the first trial started.

At the beginning of each trial, the mouse was placed in the same start chamber, and 10 s after the onset of a buzzer and light, the chamber was lifted and the mouse was free to explore the maze. The trial ended when the mouse entered the goal tunnel or after 3 min had elapsed. Immediately after the mouse entered the tunnel, the buzzer was turned off and the mouse was allowed to stay in the tunnel for 1 min. Mice were trained with 4 trials per day for 4 days. Trials were separated by 15 min. After each trial, the entire maze was cleaned with 1% incidin solution and the maze was rotated to eliminate the use of intramaze cues. Trials were recorded by using computerized tracking/image analyzer system (video camcorder: 1/3 in. SSAMHR EX VIEWHAD coupled with computational tracking system: TiBeSplit). The parameter “time to escape into the tunnel” was recorded and used for correlations with protein data.

One day after the acquisition phase, subjects received a probe trial for 90 s to check the short-retention memory. During probe trial, the tunnel leading to the target box was closed. Mice were allowed to explore the maze and latencies to reach the target hole for the first time were recorded.

On day 12, subjects again performed a probe trial for 90 s to check long-term retention memory. Mice were not trained during the time period between day 5 and 12.

Yoked controls were not trained but remained in the BM under the identical conditions without having access to the escape hole.

Multiple T-Maze (MTM). In this spatial learning task, animals learn to find the goal box based on their memory of previously visited arms. The MTM was constructed of wood and consisted of a wooden platform with seven choice points and the dimensions 150 cm × 130 cm × 15 cm and a path width of 8 cm.

Prior to testing, mice were deprived of food for 16 h to motivate food searching. Mice were placed in a start box in a black cylindrical start chamber. After 10 s elapsed, the chamber was lifted and the first trial was started. Mice were searching for the reward and the trial was completed when mice had reached the goal box or, if failed, after 5 min. Arriving in the goal box, mice were allowed to consume a small piece of a food pellet as provided reward and transferred to their home cage. Immediately after each trial, the entire maze was cleaned with 1% incidin solution. After testing, animals were given food as per body weight (120 g/kg) into the home cage, representing the amount to preserve their body weight but keeping them hungry for the following day for MTM tests. Mice were trained with 3 trials per day for 4 days. Trials were carried out using 20 min intervals.

Trials were recorded using the computerized tracking/image analyzer system as given above. The system provided the following parameters, correct or wrong decisions (wrong means a path ending), path length, speed and latency to reach the goal box. The latencies from the probe trial at day 12 were used for correlation with protein data.

On the fifth experimental day (short-term retention memory), subjects were undergoing a probe trial for 5 min. Mice were allowed to explore the maze and path length, time to reach the goal and correct and wrong decisions were recorded.

On day 12, subjects again performed a probe trial for 5 min to check long-term retention memory. Mice were not trained during the time period between day 5 and 12.

Yoked controls (20 per group, total n = 40) were placed into the BM or the MTM to remain the same time as their trained mates, but without a hole in the BM or food provided in the MTM. Since animals were exposed to the same spatial cues, but without a hole or food, mice did not develop an association between the extra-maze cues and the location of the hole or food.

Protein Studies. Six hours after the probe trials in the BM and MTM, mice were sacrificed by neck dislocation and hippocampi were dissected. Tissue samples were immediately frozen in liquid nitrogen and stored at −80 °C until used for analysis. All efforts were made to minimize animal suffering and the number of animals used.

Experiments were done under license of the federal ministry of education, science and culture, which includes ethical evaluation of the project (Project: BMWF-66.009/0152-C/GT/2007). Housing and maintenance of animals were in compliance with European and national regulations.

Sample Preparation. Individual mouse hippocampi were homogenized and suspended in 1.2 mL of sample buffer (20 mM Tris, 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 10 mM 1,4-dithioerythritol, 1 mM EDTA, 1 mM PMSF, 1 tablet Complete from Roche Diagnostics, and 0.2% (v/v) phosphatase inhibitor cocktail from Calbiochem). The suspension was sonicated on ice for approximately 30 s and centrifuged at 15 000 × g for 120 min at 12 °C. Desalting was carried out with an Ultrafree-4 centrifugal filter unit with a cutoff molecular weight of 10 kDa (Millipore, Bedford, MA) at 3000 × g at 12 °C until the eluted volume was about 4 mL and the remaining volume reached 100–200 µL. The protein content of the supernatant was determined by the Bradford assay.

Two-Dimensional Gel Electrophoresis (2-DE). Samples of 700 µg of protein were subjected to immobilized pH 3–10 nonlinear gradient strips. Focusing started at 200 V and the voltage was gradually increased to 8000 at 4 V/min and kept constant for a further 3 h (approximately 150 000 Vh totally). Prior to the second-dimensional run, strips were equilibrated twice for 15 min with gentle shaking in 10 mL of SDS equilibration buffer (50 mM pH 8.8 Tris-HCl, 6 M Urea, 30% (v/v) glycerol, 2% (w/v) SDS, trace of bromophenol blue). DTT (1%) (w/v) was added at the first incubation for 15 min and 4% iodoacetamide (w/v) instead of DTT at the second incubation step for 15 min. The second-dimensional separation was performed on 10–16% gradient SDS-PAGE. After protein fixation for 12 h in 50% methanol and 10% acetic acid, the gels were stained with colloidal Coomassie blue (Novex, San Diego, CA) for 8 h and excess of dye was washed out from the gels with distilled water. Molecular masses were determined by
running precision protein standard markers (Bio-Rad Laboratories, Hercules, CA), covering the range of 10–250 kDa. Isoelectric point values were determined as given by the supplier of the immobilized pH gradient strips.

Quantification of Protein Levels. Protein spots from each gel were outlined (first automatically and then manually) and quantified using the Proteomweaver software (Definiens, Munich, Germany). The percentage of the volume of the spots representing a certain protein was determined in comparison with the total proteins present in the 2-DE gel. The software used also revealed that spots evaluated did not contain other proteins. Moreover, only well-separated spots were considered for quantification. Only those proteins (spots) with different levels between mouse hippocampi from the BM and the MTM were further studied and were outlined and quantified in the yoked groups.

Analysis of Peptides by Nano-LC-ESI-(CID/ETD)-MS/MS (High Capacity Ion Trap, HCT). Twenty-eight spots from each group which showed different levels between the BM group and the MTM group were manually excised and placed into 0.5 mL of loblind Eppendorf tubes. Gel plugs were washed with 10 mM ammonium bicarbonate and 50% acetonitrile (ACN) in 10 mM ammonium bicarbonate repeatedly. Addition of 100% ACN resulted in gel shrinking and the shrunk gel plugs were then dried in a Speedvac Concentrator 5301 (Eppendorf, Germany). The dried gel pieces were reswollen and in-gel digested with 40 ng/µL trypsin (Promega, Madison, WI) in digestion buffer (consisting of 5 mM octyl b-D-glucopyranoside (OGP) and 10 mM ammonium bicarbonate) and incubated overnight at 37 °C. Peptide extraction was performed with 10 µL of 10 mM ammonium bicarbonate overnight, 15 µL of 1% formic acid (FA) in 5 mM OGP for 30 min, 15 µL of 0.1% FA for 30 min, and subsequently 0.1% FA in 20% ACN for 30 min. The extracted peptides were pooled for HCT analysis.

A total of 40 µL of extracted peptides was analyzed by HCT. The HPLC used was a biocompatible Ultimate 3000 system ( Dionex Corporation, Sunnyvale, CA) equipped with a PepMap100 C-18 trap column (300 µm x 5 mm) and PepMap100 C-18 analytic column (75 µm x 150 mm). The gradient was (A = 0.1% FA in water, B = 0.08% FA in ACN) 4–30% B from 0 to 105 min, 80% B from 105 to 110 min, 4% B from 110 to 125 min. The flow rate was 300 nL/min from 0 to 12 min, 75 nL/min from 12 to 105 min, 300 nL/min from 105 to 125 min. A HCT ultra PTM discover system (Bruker Daltonics, Bremen, Germany) was used to record peptide spectra over the mass range of m/z 350–1500, and MS/MS spectra in information-dependent data acquisition over the mass range of m/z 100–2800. Repeatedly, MS spectra were recorded followed by three data-dependent CID MS/MS spectra and three ETD MS/MS spectra generated from three highest intensity precursor ions. An active exclusion of 0.4 min after two spectra was used to detect low-abundance peptides. The voltage between ion spray tip and spray shield was set to 1100 V. Drying nitrogen gas was heated to 170 °C and the flow rate was 10 L/min. The collision energy was set automatically according to the mass and charge state of the peptides chosen for fragmentation. Multiple charged peptides were chosen for MS/MS experiments due to their good fragmentation characteristics. MS/MS spectra were interpreted and peak lists were generated by DataAnalysis 3.4 (Bruker Daltonics, Bremen, Germany). Searches were done using the MASCOT 2.2.04 (Matrix Science, London, U.K.) against latest UniProtKB database for protein identification. Searching parameters were set as follows: enzyme selected as trypsin with two maximum missing cleavage sites, species limited to mouse, a mass tolerance of 0.2 Da for peptide tolerance, 0.2 Da for MS/MS tolerance, fixed modification of carbamidomethyl (C) and variable modification of methionine oxidation and phosphorylation (Tyr, Thr, and Ser). Positive protein identifications were based on a significant MOWSE score. After protein identification, an error-tolerant search was done to detect nonspecific cleavage and unassigned modifications. Protein identification and modification information returned from MASCOT were manually inspected and filtered to obtain confirmed protein identification and modification lists of CID MS/MS and ETD MS/MS.16

Only proteins that were not showing peptides from other proteins were considered. Phosphatase treatment to verify the phosphorylation at T509 was carried out as described previously.17

Western Blotting. Aliquots of samples for 2DE were used for Western blotting in order to verify results obtained from 2-DE quantification of hippocampal proteins.

Samples prepared as described above were loaded onto 12.5% ExcelGel SDS homogeneous gels (GE Healthcare, Buckinghamshire, U.K.). A total of 5 or 10 µg of samples was loaded. Electrophoresis was performed with the Multiphor II Electrophoresis System (Amersham Pharmacia Biotech, Uppsala, Sweden). Proteins separated on the gel were transferred onto PVDF membranes. Membranes were incubated with diluted rabbit anti-VHR dual-specific phosphatase antibodies (1:1000; Cell Signaling Technology, Inc.), rabbit monoclonal antibody against calmodulin (1:5000) and goat anti-Voltage dependent anion channel 2 (1:2000) (both from Abcam plc, Cambridge, U.K.) and detected with horseradish peroxidase-coupled secondary antibodies anti-rabbit IgG (Cell Signaling Technologies, Inc.) and anti-goat IgG (Abcam plc, Cambridge, U.K.) according to the supplier’s protocol.

Membranes were developed with the Amersham ECL Plus Western Blotting Detection System (GE Healthcare, Buckinghamshire, U.K.). Densities of immunoreactive bands were measured by Image J software program (http://rsb.info.nih.gov/ij/).

Statistical Analysis. Statistical analysis to reveal between-group differences (task-specific protein) was performed by Mann–Whitney-U-test. Correlation between latencies and protein levels were calculated according to Pearson Correlation analysis. A probability level of P < 0.05 was considered as statistically significant. All calculations were performed using SPSS version 14.0 (SPSS, Inc., Chicago, IL).

Results

Mouse hippocampal proteins from four groups, mice trained in the BM, MTM, and their corresponding untrained yoked controls, were separated on 2-DE and an average of 464 well-separated spots were quantified using specific software. Spots that were significantly different were identified using nano-LC-ESI-MS/MS. Levels of 28 spots representing 24 different proteins were different between the BM and the MTM and were linked to spatial memory (Figure 1). Identification data including MS/MS peptides are listed in Supplementary Table 1. The 28 spots that were different between the BM and the MTM were corrected by quantification in the yoked controls to exclude possible changes in hippocampal expression due to the effect of stress or food restriction per se. The quantification results of significantly different protein levels are demonstrated in Table 1.
Pearson correlation analysis showed that heat shock 70 kDa protein 2 revealed threonine 509 phosphorylation in both the BMW and MTM groups. The site assignments of PTMs of the 24 differentially expressed proteins were investigated using nano-LC-ESI-(CID/ETD)-MS/MS. The PTMs revealed by CID and ETD were partly overlapping. The identification as well as PTM characterizations of 28 spots with HCl are shown in Supplementary Table 3. The site assignments of PTMs are demonstrated in Table 2.

As observed in Table 1, four proteins showed higher levels in the BM and 20 showed higher levels in the MTM. No statistically significant differences were observed between the BM yoked group versus the MTM yoked group.

Pearson correlation analysis showed that heat shock 70 kDa protein 4L and fatty acid-binding protein (epidermal) positively correlated with latency to reach the goal in the MTM (Supplementary Table 2).
### Table 2. Post-Translational Modifications of Mouse Hippocampal Proteins

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<th>acc. no.</th>
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<td>P52480</td>
<td>Pyruvate kinase isozymes M1/M2</td>
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groups providing experimental evidence for the prediction “by similarity” in UniProtKB database (http://www.expasy.org/uniprot/P63101). The mass shift was reversible following phosphatase treatment of the spot. The MS/MS spectra of PTM assignments are shown in Supplementary Figure 1. Phospho-serine/threonine treatment of the spot. The MS/MS spectra of PTM modifications in the Barnes maze

<table>
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<td>Vesicle-fusing ATPase</td>
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Discussion

The major findings of the study are task dependence of hippocampal levels of several proteins from individual protein pathways and cascades probably reflecting learning and memory formation in two land mazes. Second, these hippocampal proteins could be unambiguously assigned to spatial memory.

In a recent publication, different performance of C57BL/6J in the MTM and in the BM was reported and hippocampal tissue was obtained from this mouse strain 6 h following the probe trials at day 12. Differences at the functional level in the two land mazes made us investigate hippocampal protein levels and indeed, hippocampal protein levels of a series of proteins, that is, the hippocampal expression pattern, was different. The use of yoked controls that were not trained for spatial memory performance (see above) allowed to fairly assign a role of these 24 proteins to spatial memory (Table 1). Most proteins have not been linked to memory formation so far at all and herein a role in spatial memory formation is proposed.

As shown in Results, 20 of these proteins demonstrated higher levels in the MTM while four were presenting with lower levels in the MTM.

Changes in metabolically related proteins—and mainly carbohydrate handling enzymes were observed—may be due to the fact that mice were to some extent food deprived in the MTM but not in the BM, although modifications of metabolically related proteins were also detected in and linked to spatial memory in the MWM. Also differences between chaperones heat shock 70 kDa protein 4L and fatty acid-binding protein as well. expression forms of this protein and this holds for correlations for heat shock 70 kDa protein 4L is, however, challenged by the significant correlation between latency at day 12 with both expression forms of this protein and this holds for correlations between the fatty-acid binding protein as well.

The individual isoforms of dihydropyrimidinase-related proteins 2 and 4 could be linked to spatial memory herein and to synaptic plasticity in previous work. Learning and memory formation in the MTM could have involved synaptic plasticity that definitely occurred at day 12 differently than in the BM.
Likewise, ferritin may play a role in spatial memory as it was reported to be associated with synaptic function and different levels between the two spatial tasks may reflect different synaptic activation or handling.

The cytoskeletal proteins actin-related protein 2/3 complex, actin-related protein 3, cofilin-1 and F-actin capping protein subunit beta are known to be involved in spine and synapse formation and vesicular trafficking and F-actin-capping protein is one of the key proteins for protein–protein interactions in spatial memory by synapse formation and reorganization of growth cones during the consolidation phase of memory. Myotrophin (synonym: protein V-1) is linked to monoaminergic neural transmission and regulates actin polymerization that would be in agreement with synaptic plasticity and with memory formation.

Although a series of transcription factors (TF) have been shown to be linked to the process of spatial memory formation, no information on the transcriptional activator protein Pur-alpha in memory has been reported so far, but a role for this TF in hippocampal synaptic plasticity following exercise was proposed. Another two proteins from the protein synthetic machinery, 60S acidic ribosomal protein P2 and vesicle-fusing ATPase, were linked to spatial memory and presented with different hippocampal levels. While no information so far is available for any role in memory formation, synaptic plasticity or neurogenesis for the 60S acidic ribosomal protein P2, vesicle-fusing ATPase (synonym: N-ethylmaleimide-sensitive fusion protein, NSF) is an essential constituent of the exocytotic machinery and thus is important in regulating neural transmission. Different hippocampal NSF levels observed between the two land mazes may represent changes in basic neural transmission mechanisms at the synaptic level.

Levels of three major signaling proteins were different by quantifying 2DE gels between the MTM and the BM and results were verified by immunohblotting:

- Voltage-dependent anion-selective channel protein 2 (VDAC2), a constituent of synaptosomal associated proteins, showed higher levels in the BM and a task-specific role is proposed.
- Calmodulin mediates control of a key enzyme in spatial memory formation, Ca²⁺-calmodulin kinase II, and showed higher levels in hippocampi of mice trained in the MTM in the current study. It is intriguing that levels of this major determinant of spatial memory were task-specific.
- Dual-specificity protein phosphatases 5 and 6 were proposed as spatial memory-linked genes at the nucleic acid level and herein we add dual-specificity protein phosphatase 3 as a task-specific and spatial memory-related protein in the mouse.

Taken together, proteins from several protein pathways and cascades were linked to spatial memory and their levels were different between the MTM and the BM.

These data may help to interpret data from previous work on spatial memory using these mazes and assist in the design of future studies on spatial memory at the protein level. To the best of our knowledge, this is the first report to show task-dependent protein levels in two independent paradigms for the evaluation of spatial memory. Further work on proteins linked to spatial memory formation will be carried out. This work will include immunohistochemistry to show differences of protein levels in subareas and individual cell types of the hippocampus, their corresponding mRNA levels by in situ hybridization and further work on post-translational modifications, known to be required for memory formation.
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Supporting Information Available: Supplementary Figure 1, MS/MS spectra of T509 phosphorylation revealed by nano-LC-ESI-(CID/ETD)-MS/MS. Phosphorylation of dihydro-7-propimidinidase-related protein 2 after phosphatase treatment. A mass shift of 80 indicating phosphorylation was disappearing following phosphatase treatment (below). Distance between dashed lines or, between y2 and y3, and b12 and b13 is provided representing dephosphorylation. Supplementary Table 1, protein identification of mouse hippocampal proteins with different levels between the BM and MTM by nano-ESI-LC-MS/MS. Supplementary Table 2, Pearson Correlations between protein level between y2 and y3, and b12 and b13 is provided representing dephosphorylation. Supplementary Table 3, identification and post-translational modification characterization of mouse hippocampal proteins differentially expressed between BM group and MTM group with nano-LC-CID-MS/MS and nano-LC-ETD-MS/MS, respectively. This material is available free of charge via the Internet at http://pubs.acs.org.

References


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