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Cognitive performance and age-related changes in the hippocampal proteome

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Abstract

Declining cognitive performance is associated with increasing age, even in the absence of overt pathological processes. We and others have reported that declining cognitive performance is associated with age-related changes in brain glucose utilization, long-term potentiation and paired-pulse facilitation, protein expression, neurotransmitter levels, and trophic factors. However, it is unclear whether these changes are causes or symptoms of the underlying alterations in dendritic and synaptic morphology that occur with age. In this study, we examined the hippocampal proteome for age- and cognition-associated changes in behaviorally stratified young and old rats, using 2-DIGE and MS/MS-MS. Comparison of old cognitively intact with old cognitively impaired animals revealed additional changes that would not have been detected otherwise. Interestingly, not all age-related changes in protein expression were associated with cognitive decline, and distinct differences in protein expression were found when comparing old cognitively intact with old cognitively impaired rats. A large number of protein changes with age were related to the glycolysis/gluconeogenesis pathway. In total, the proteomic changes suggest that age-related alterations act synergistically with other perturbations to result in cognitive decline. This study also demonstrates the importance of examining behaviorally-defined animals in proteomic studies, as comparison of young to old animals regardless of behavioral performance would have failed to detect many cognitive impairment-specific protein expression changes evident when behavioral stratification data was used.

Keywords

protein; hippocampus; behavior; aging; cognitive decline

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Even in the absence of overt pathological processes, cognitive performance decreases with age in both humans and animal models. In humans, this decline in function clearly diminishes quality of life, is a major factor in loss of independence, and imparts a costly burden to society as a whole, and to the medical health care system in particular. For many years, diminished cognitive function was believed to be, at least in part, a reflection of neuron loss across the lifespan. More recent studies, however, utilizing contemporary stereological quantification techniques, have provided compelling evidence that such neuron loss rarely takes place during the course of normal, healthy aging (Hof and Morrison, 2004). Therefore, the age-related decline in cognitive function must be, of necessity, a reflection of a decrease in effective neural processing and dysregulation of neuronal and glial function.

The underlying mechanisms for the decrease in neural processing as the brain ages remain to be determined, but undoubtedly include changes at the molecular, cellular, and/or structural levels. To date, studies have demonstrated age-related changes in glucose metabolism, electrophysiological correlates of learning and memory (long-term potentiation and paired-pulse facilitation), protein expression (synaptophysin, calcium regulatory proteins, excitatory and inhibitory neurotransmitter receptors), neurotransmitter levels (dopamine, norepinephrine, 5-HT, GABA, acetylcholine) as well as several trophic factors (BDNF, NGF, IGF-1) (Baxter et al., 1999; Smith et al., 2000; Mora et al., 2007; Gavilan et al., 2007; Majdi et al., 2007; Foster, 2007; Shi et al., 2007; Hwang et al., 2006; Rowe et al., 2007; Gant et al., 2006). Considerable evidence also exists for structural changes as the brain ages e.g. age-related atrophy of various aspects of dendritic and synaptic morphology as well as vascular rarefaction (Dickstein et al., 2007; Sonntag et al., 1997; Sonntag et al., 2000; Shi et al., 2005). Despite the advances in our understanding of age-related changes that occur in the brain, the etiology of cognitive impairment with age remains unknown.

Interestingly, recent behavioral studies have revealed that, as in humans, aging animals can be subdivided into cognitively-intact and cognitively-impaired categories [see review by (Gallagher and Rapp, 1997)]. Cognitively intact animals show few cognitive changes with age compared to young animals while cognitively impaired animals demonstrate reduced performance on several tasks of learning and memory. Such classifications have been used to facilitate the identification of the specific neurobiological mechanisms of cognitive impairment (Brightwell et al., 2004; Gallagher et al., 2003; Rowe et al., 2007; Majdi et al., 2007). Importantly, such analyses become critical for separating the effects of normal age-related changes that occur in the brain and are unrelated to cognitive decline from those that contribute to mild cognitive impairment.

Delineating the specific molecular mechanisms that occur with healthy aging from those associated with cognitive decline is essential for development of targeted prevention and intervention therapeutics. Although analysis of gene expression changes have revealed important information regarding the molecular basis of cognitive decline, alterations in gene expression are not always associated with changes at the protein level. The application of proteomic analyses to neurobiological studies of aging is becoming more common as the field adopts more systems-oriented approaches (Butterfield et al., 2006; Carrette et al., 2006; Poon et al., 2004). Proteomic studies of the hippocampus using models relevant to aging or in aging animals have revealed regional dysregulation of processes including metabolism, glutamate processing and protein synthesis (Poon et al., 2006b), protein processing (Gavilan et al., 2006), cytoprotection and apoptosis (Calabrese et al., 2004), and synaptic connectivity and functionality (Head et al., 2007). Previous proteomic studies have not, however, behaviorally assessed subjects to distinguish between more general effects of aging on protein expression and protein changes associated specifically with cognitive impairment.

In this study, we assessed performance of young and aged animals in a hippocampally-dependent behavioral task of learning and memory and separated animals into cognitively intact and cognitively impaired categories. A proteomics approach (2-DIGE, 2 dimensional difference gel electrophoresis) (Alban et al., 2003; Tonge et al., 2001) was then used to identify proteins changed with age and/or cognitive impairment in protein isolated from the hippocampus. Proteins statistically regulated with aging or cognitive impairment were imported into pathway analysis software to identify potential pathways over-represented by protein expression changes.

Materials and methods

Animals and behavioral testing

Fisher 344xBrown Norway (F1) male rats (10 and 27 months of age) were obtained from the NIA colony at Harlan Industries (Indianapolis, IN). Animals were singly housed in polycarbonate cages with food (Purina Mills Richmond IN) and water available ad libitum. The animal rooms were kept at a constant temperature and humidity and maintained on a 12:12 light dark cycle. The animal facilities were fully accredited by the American Association for Accreditation of Laboratory Animal Care and all procedures were approved by the Institutional Animal Care and Use Committee.

After adaptation to our animal facility for two weeks, acquisition was assessed using a variation of the Morris Maze (Morris et al., 1982). The water maze employed in this study was a galvanized metal tank 1.7m in diameter and 0.6m in height. Water was added to the tank to a depth of 25cm, and made opaque through the addition of non-toxic, water-based white paint. The escape platform surface was 1–2cm below the surface of the water. Performance in the water maze was recorded by an automated tracking system (Noldus Ethovision 3.0, Wageningen, Netherlands), which acquired images from a camera mounted 1.6m above the surface of the water. Place discrimination trials were designed to evaluate the rate and extent of acquisition of the spatial task. Animals were given one trial (60 seconds) each day for 15d with the platform located in the same position across all days of testing. At the beginning of the trial, the animal was placed in the maze, from one of three start locations, facing the outside of the pool. To escape, the animal was required to find the platform within 60 sec. A curtain surrounded the pool and visual objects were positioned at various locations to serve as cues for the location of the platform. Latency to find the platform (in seconds) was the dependent measure with shorter latencies indicating better performance. On day 16, a Probe Trial was conducted during which the escape platform was removed. The animal was placed in the maze and cumulative distance to the platform and time spent in the Annulus-40 (the area 40cm around the previous platform location) was recorded during a 30s interval. Two days later, visual performance was assessed over 4 consecutive trials using a visible (raised) platform. Old animals exhibited a range of performance on both measures and were divided into intact and impaired categories. One week later, animals were sacrificed by decapitation without anesthesia, hippocampus rapidly dissected and frozen at -80°C until submitted for protein analysis. At sacrifice animals were examined for gross pathologies. Peripherally, animals were examined for tumors, frank kidney disease, and cardiac hypertrophy, as these diseases would compromise the behavioral performance of the animals. Gross neuropathology of the brain was also evaluated (e.g., severe atrophy of cortex or widening of the lateral ventricles) as exclusionary criteria.

2-DIGE

Protein was fractionated using the ProteoPrep Universal Extraction Kit (Sigma) according to the manufacturer's protocol. Hippocampal tissue was sonicated in 5mM Trizma and fractionated into soluble and membrane fractions. Both fractions were saved and initial analysis

was performed on the soluble fraction. Five animals each from the Young, Old-Intact, and Old-Impaired groups were analyzed using a quantitative 2-DIGE protocol similar to that previously reported (Freeman et al., 2005). All 2-DIGE methods and results were performed and are provided in a MIAPE-GE compliant form (Taylor et al., 2007), Supplemental Table 1. The soluble fraction was precipitated from other cellular substances using 2D-Cleanup (GE Healthcare, Piscataway, NJ, USA), resuspended in sample buffer (30mM TrisCl, 2M Thiourea, 7M Urea, and 2% CHAPS, 1% ASB-14, pH 8.5), and quantified using 2D-Quant (GE Healthcare). A 600µg normalization/preparative pool was created from 50µg aliquots of protein from each sample. 50µg of each sample or aliquot of the normalization pool was labeled with 400pM of the appropriate fluorophor at pH 8.5 (Figure 1). Proteins were focused according to isoelectric point using an ETTAN IPGPhor apparatus (GE Healthcare) in Immobiline DryStrip holders. Briefly, a 24cm pH 3-10NL strip (GE Healthcare) was used for isoelectric focusing and a detergent combination of 2% CHAPS and 1% ASB-14 was used to improve solubility. Strips were overlaid with mineral oil to prevent drying and the ETTAN IPGPhor apparatus was set to 20°C. Focusing used the following voltages: 14h at 0V (rehydration); 6 h at 30V (rehydration); 3h at 300V (step-and-hold); 3h at 600V (gradient); 3h at 1000V (gradient); 3h at 8000V (gradient); 4h at 8000V (step-and-hold). Each of the strips were then equilibrated to SDS in 10 ml 50mM TrisCl, 6M Urea, 30% glycerol, 2% SDS, and 0.5% dithiothreitol for 15 min. A second equilibration was performed as above but with 4.5% iodoacetamide instead of dithiothreitol. Focused proteins were then separated by molecular weight on 12.5% polyacrylamide gels (26 cm-w 20 cm-h 1.0 mm-thick) in an Ettan DALT 12 (GE Healthcare) gel apparatus. The IEF strips were placed on top of the two dimensional gel and sealed with a 0.5% low melting point agarose solution containing bromophenol blue. The 50mM TrisCl, 384mM glycine, 0.2% SDS running buffer was placed in the upper reservoir and 25mM TrisCl, 192mM glycine, 0.1% SDS was used in the lower reservoir. Gels were run at 1.5W/gel overnight with the temperature controlled at 20°C.

For the nonquantitative preparative gel (the gel to be used later for spot picking and protein identification), one of the glass plates was labeled with picking reference stickers and the plate was then treated with 80% ethanol, 0.02% glacial acetic acid, and 0.001% bind-silane and allowed to dry before pouring of the gel. After completion of electrophoresis, the preparative gel was fixed for 1h with 30% methanol and 7.5% glacial acetic acid. The preparative gel was then stained for 6h with SyproRuby (BioRad, Hercules, CA, USA) and washed with 10% methanol and 6% glacial acetic acid for 2h. The quantitation gels, those with the Cy dye-labeled proteins, were not bound to the plate because post-staining of the gels was unnecessary.

All two-dimensional gels were scanned on a Typhoon 9410 (GE Healthcare) fluorescent imager at a resolution of 100 µm. Photomultiplier tube voltages were individually set for each of the three color lasers to ensure maximum, linear signal. The same voltages were then used for all gels. The preparative gel was scanned at 100µm with just one laser. Gel images were then imported into DeCyder 6.5 (GE Healthcare). Spot detection was conducted and spot exclude filters (based on peak slope, area, and height) act automatically. This was followed by manual inspection to eliminate other, artifactual spots and to re-include spuriously eliminated spots. Spot maps (all of the spots in a gel and their locations) were then matched automatically based on landmarked, manually matched spots. Spot matching was then checked manually. Quantitation of spots was accomplished by creating a ratio of each Cy3 and Cy5 channel to the normalization pool/Cy2 channel present on each gel. Samples from each group were randomly assigned to the Cy3 or Cy5 channel to ensure no dye-based artifacts in quantitation (Figure 1). ANOVA was then applied to identify differentially regulated spots.

Protein identification/mass spectrometry

Protein identification methods are provided below and in a MIAPE-MS compliant form in Supplemental Table 1 (Taylor et al., 2007). Spots were robotically picked from the preparative gel using an Ettan Spot Picker (GE Healthcare). The spots chosen were determined on the basis of differential expression from the 2-DIGE analysis, and a random sampling of proteins that were unchanged was also picked for identification. The picker head was calibrated using the reference stickers placed on the preparative gel plate. Excised gel plugs were automatically placed in 96-well, barcoded plates and subjected to in-gel trypsin digestion.

Plugs were washed twice with 200mM ammonium bicarbonate/40% acetonitrile for 30 minutes at 37°C and then dried with 75% acetonitrile placed in a speedvac. 20μL of 20μg/mL trypsin in 100μM HCl, 36mM ammonium bicarbonate/8% aAcetonitrile was then added and the plugs incubated overnight at 37°C. Peptides were extracted with 50% acetonitrile/0.1% TFA and desalted and concentrated using C18 ZipTips (Millipore, Billerica, MA, USA).

Peptides were spotted onto an OptiToF 384-position MALDI plate insert (Applied Biosystems) in 1.0μL volumes and overlaid with 0.7μL of α-Cyano-4-hydroxycinnamic acid (ACH-cinnamic acid) matrix (Amersham Biosciences) in 50:50 acetonitrile:ddH₂O with 6mM ammonium phosphate. Peptides were then analyzed by MALDI-ToF/ToF mass spectrometry using a 4800 Proteomics Analyzer (Applied Biosystems, Foster City, CA, USA), calibrated with Applied Biosystems 4700 Proteomics Calibration Mix, in the Penn State Mass Spectrometry Facility. For each sample, an initial mass spectrum was collected. Measurements were collected in the positive ion reflectron mode between 800 and 4000 m/z with a signal-to-noise filter of 10, mass exclusion tolerance of 0.2Da, and a peak density filter of 50 peaks per 200Da. Based on the initial mass spectrum, up to 15 precursors were selected for tandem mass spectrometry (MS/MS) analysis, excluding those included on an exclusion list containing trypsin autolysis, matrix, and tryptic peptides of human keratin, as well as those identified in a “blank” gel plug. MS/MS was performed without collision-induced decay in a mass range from 60Da to 20Da below the precursor mass with a fragment tolerance of 0.2Da for +1 charged ions. Using GPS Explorer 3.6 software (Applied Biosystems), the MS and MS/MS data were submitted to a MASCOT (v2.0.00) search engine for identification. The NCBI nonredundant database with the *Rattus* taxonomy (downloaded Feb 16, 2008, 107,758 entries searched) and a concatenated, reversed ‘decoy’ version were used for the searches with a mass accuracy of 50ppm, 1 missed trypsin cleavage, fixed carbamidomethylation of cysteine residues and variable oxidation of methionine residues. A protein was considered identified if the MASCOT confidence interval was >99th percentile and those proteins with a MASCOT confidence interval <99% were excluded from the subsequent analyses.

Statistical and Bioinformatic Analyses

Behavioral data were analyzed by repeated ANOVA followed by the Newman-Keuls post hoc procedure ($\alpha=0.05$). Differential protein expression was determined by a One-Way ANOVA with false-discovery rate (FDR) using DeCyder 6.5. Principle component analysis by group was performed using GeneSpring GX 7.3 (Agilent Technologies) and was mean centered and scaled. Ontological groupings were analyzed with the PANTHER ontology database (Applied Biosystems) (Thomas et al., 2003; Mi et al., 2005) and specific ontologies overrepresented in the changes were determined by comparison to the NCBI-Rattus database ($p<0.05$). To examine potential networks or pathways of proteins regulated by age or cognitive status, expression levels and protein names were imported into the Ingenuity Pathway Analysis (IPA) system (Ingenuity Systems, Redwood City, CA) to visualize networks of proteins or canonical pathways. Pathways were considered significantly regulated with a $p<0.05$, Fisher’s Exact Test.

Results

Behavioral results

Animals were subdivided from a cohort of 15 animals (5 Young and 10 Old animals) based on their performance on the training blocks and probe trial phases of the Morris Water Maze and the absence of gross, peripheral underlying age-related pathology (i.e., tumors, cardiovascular hypertrophy, enlarged kidneys) observed at necropsy. This assessment was necessary since the presence of peripheral disease may influence behavioral results. The absence of significant gross pathology in the experimental animals in the present study was supported by the stability of body weight in these animals in the weeks before and during behavioral testing (data not shown) which we have found to be a sensitive marker of disease in aged rodents. Additionally, animals were evaluated for frank neuropathology, including pituitary tumors, widening of the lateral ventricles, and severe cortical atrophy. No animals included in this study exhibited gross peripheral or neuropathological characteristics evident at necropsy. Based on the data from the probe trials, old animals were divided into Old Intact and Old Impaired groups. Data in Figure 2A and 2B represent five animals/group categorized as Young Cognitively Intact, Old Cognitively Intact, and Old Cognitively Impaired animals and used in the subsequent proteomic analyses. Old animals with behavioral performance in the same range as the Young animals were categorized as Old Cognitively Intact, and those with behavioral performance below the range of Young animals were placed in the Old Cognitively Impaired group. For the behavioral analysis, data representing all three start locations was summed over a three day period into one of 5 Training Blocks (representing the 15 trials).

In Young and Old Cognitively Intact animals, latency to reach the platform improved over the training sessions (Figure 2A; lower numbers representing better performance) and no differences were evident between these two groups (overall group effect $p=0.289$). However, Old Cognitively Impaired animals showed no improvement in latency to reach the platform during the first 3 blocks of training. Latency of the Old Impaired animals to reach the platform was significantly greater in block 2 ($p=0.044$) compared to Young animals and greater than Young or Old Intact animals during block 3 ($p=0.005$ and $p=0.017$, respectively). Similar results were obtained from measurements of the distance traveled to the escape platform (Supplemental Figure 1).

Analysis of the Annulus-40 time during the Probe Trial also revealed decreased performance in a subset of the Old animals. Young and Old Intact animals spent significantly greater time in the Annulus-40 around the platform during the 30 second trial compared to Old-Impaired animals (Young Intact vs Old Impaired $p=0.012$ and Old Intact vs Old Impaired $p=0.016$) (Figure 2B).

Analysis of sensorimotor function measured during the acquisition phase of the experiment as velocity (cm/sec) to reach the platform or performance during the visible platform trials conducted after cognitive testing revealed no differences between groups (data not shown).

Proteomic results

Labeled proteins were separated and produced typical spot patterns for a two-dimensional gel (see Figure 3 for example image). 1,123 spots were matched across gels. The protein spot patterns demonstrated high resolution and a large number of spots across the pH and molecular weight ranges (Figure 3). The eight quantitation gels and the preparative gel produced highly similar spot patterns. Protein spots were detected from gel images by DeCyder 6.5 software and matched automatically across all seven gels. After manual checking of the spot matching, 918 spots were matched across at least six gels. The Cy2-normalized signal intensities were used to test for potential differences between groups.

Protein identification was performed by robotic gel excision of selected spots that represented both statistically significant changes (as described below) as well as a selection of proteins that were not changed by condition, and MALDI-ToF/ToF MS and MS/MS analysis. 190 spots were identified with a high level of confidence (MASCOT confidence interval >99%, Supplemental Table 2). Example parent MS and peptide precursor MS/MS spectra, and the resulting amino acid sequence identification, are depicted in Figure 4. To aid in the identification of biological processes that may be affected by the proteomic changes, ontological analysis of identified proteins with expression regulated with age and/or cognitive status was performed using the PANTHER ontology database (www.pantherdb.org) (Thomas et al., 2003; Mi et al., 2005). Protein expression changes with age (Young Intact vs. Old Intact) belonged to a number of processes including carbohydrate metabolism, cell structure and signal transduction, while expression changes related to cognitive impairment (Old Intact vs Old Impaired) included protein and carbohydrate metabolism (Table 1 and Table 2, respectively). A full list of protein spots (both those identified and unidentified by mass spectrometry) regulated with age and/or cognitive status is presented in Supplemental Table 3.

Statistical analysis of expression changes for identified proteins was conducted in two ways. In the first approach, age-related changes were determined by comparing Old animals regardless of cognitive performance against the Young animals (Young vs Old) (left circle, Figure 5). Cognitive status-specific changes were determined by comparing Old Impaired animals against Old Intact animals (right circle, Figure 5). Nine identified proteins were statistically different between groups of Old animals behaviorally phenotyped according to cognitive performance (Old Intact vs Old Impaired, Table 2). The age-based comparison of Young and Old animals reflected 19 identified protein changes (Table 1), with only one protein common between the two comparisons. The second approach limited age-related changes to those differences between Young Intact and Old Intact animals, and compared age-specific and cognitive status-specific changes with each other as well as to the analysis of Young Intact vs Old Impaired animals, which combines the effects of both increased age and decreased cognitive status (Supplemental Figure 2). Overlap in protein expression changes detected by these comparisons was limited, with only one protein common to both age-specific and cognitive status-specific changes and several proteins evident in only one analysis. The comparison of Young Intact vs. Old Impaired animals reflects several changes associated with aging and cognitive impairment, but lacks the specificity of the comparisons restricted to either age or cognitive status alone.

To examine how the proteomic profiles, in total, of the different animal groups relate to each other, a Principal Component Analysis (PCA) by condition was performed. In this analysis the union of all potential protein expression changes from the statistical analyses was used to cluster the animal groups (Young Intact, Old Intact, Old Impaired). The groups segregate according to two components (Figure 6). The first component separated the animal groups by age in the horizontal with both Old groups segmenting from the Young group (x-axis, 1st component, 70.9% of the study variance). The second component separated sample groups by cognitive status in the vertical with Young Intact and Old Intact groups segregating from the Old Impaired animal group (y-axis, 2nd component, 29.1% of study variance). This visualization further demonstrates that there are two proteomic shifts occurring in the experimental groups, one with age and a second with cognitive status. Furthermore, when comparing the Old Impaired and Young Intact groups, the effects of both age and cognitive status are evident. The larger amount of study variance accounted for by the first component (Age) indicates that, in total, the age-related proteome changes are larger in number and higher in magnitude than the proteome changes specifically associated with cognitive performance.

Next, Ingenuity Pathway analysis was performed and the canonical glycolysis/gluconeogenesis pathway was determined to be regulated ($p < 0.002$) based on alterations in

expression of several enzymes with aging and/or cognitive impairment (Figure 7). These changes suggest an age-related impairment of glucose, pyruvate and lactate metabolism as well as a decrease in ATP-generating metabolic capacity with age. These changes were distinct from those changes occurring specifically with cognitive impairment in old animals, in which few glycolytic proteins changed with age were associated with decreasing cognitive performance. Both the age- and cognitive performance- related changes were evident when cognition- and age-related changes were examined in combination (Young Intact vs Old Impaired).

Discussion

Age is a primary risk factor for cognitive decline in rodents, non-human primates and humans, indicated by increasingly impaired performance in tasks of learning and memory. Although the etiology of age-related cognitive decline, and specifically impaired hippocampally-dependent learning and memory, remains unclear, cognitive decline is associated with impaired function of many cellular processes, including, but not limited to, decreased glucose metabolism and blood flow as well as alterations in synaptic morphology/plasticity and electrophysiological correlates of learning and memory (Rosenzweig and Barnes, 2003; Blalock et al., 2003; Brewer et al., 2007). The segregation of results from this proteomic analysis by behavioral phenotype (as has been done for gene expression (Rowe et al., 2007)) will likely be a key factor in identifying those proteins that specifically contribute to cognitive decline with age.

Systems neurobiology of aging and cognitive decline

Previous transcription studies have detailed both age- and cognitive performance-related changes in gene expression. These studies identified cognitive status-associated gene expression patterns associated with synaptic morphology, transcription factors, and protein modification (Burger et al., 2007), as well as glucose utilization and receptor/signal transduction (Rowe et al., 2007). There are also numerous reports of changes in hippocampal expression of specific mRNAs with age. Proteomic reports have addressed age-associated changes in hippocampal protein expression and posttranslational modification (Butterfield et al., 2006; Poon et al., 2006b; Weinreb et al., 2007). Additional reports have focused on specific proteins related to synaptic transmission (Adams et al., 2008; Sato et al., 2005; Shi et al., 2005), mitochondrial function and oxidative stress (Poon et al., 2006a; Poon et al., 2006b; Squier, 2001) and proteolytic proteins (Paz et al., 2006; Nyffeler et al., 2007). These latter studies are important contributions to our understanding of age-related changes within the hippocampus but it is unclear which of the described protein changes are the result of age and which are associated specifically with age-related cognitive decline.

The Morris Water Maze is a standard testing paradigm for rodents that enables assessment of hippocampally-dependent spatial learning, and subsequent classification of subjects into “Intact” or “Impaired” functional groups for analysis of cognition-associated neurobiological changes. In this study, mature-Young (10 month) and Old (27 month) rats were classified according to their time in the Annulus-40 during the Probe Trial of the behavioral task. Young animals and a subset of Old animals spent a similar amount of time in the Annulus-40, while the remaining Old animals exhibited a reduced Annulus-40 duration indicating an age-related impairment in memory (Gallagher and Rapp, 1997). This stratification into Cognitively Intact and Cognitively Impaired categories allows the neurobiological changes with cognitive decline to be separated from those changes that occur with age regardless of cognitive performance (Brightwell et al., 2004; Gallagher et al., 2003; Rowe et al., 2007).

Importantly, a number of proteomic changes related to cognitive performance would have been obscured without this behavioral phenotyping. The limited overlap between age-specific and

cognitive status-specific changes in protein expression indicates that a variety of different processes are implicated in aging and age-related cognitive decline. While this point may appear obvious, the data presented here provide a proteomic analysis of the differences in age and cognitive performance associated hippocampal protein expression. Our results however, do not imply that age-related changes are unimportant to cognitive decline since age-related changes may be risk-factors or initiating events that, when combined with additional perturbations, result in cognitive impairment.

To understand the potential impact of the proteomic changes observed, proteins altered either with aging or cognitive performance were analyzed for biological process ontologies using the PANTHER ontology database. Ontological analysis indicated that proteins from a variety of processes including glycolysis and carbohydrate metabolism, were altered with aging and cognitive decline. These data agree with previous functional genomic studies of the hippocampus of aged rats (Rowe et al., 2007; Blalock et al., 2003). To extend our findings, Ingenuity Pathway analysis indicated that several protein changes with aging are related to the glycolysis/gluconeogenesis pathway as described in detail below.

Specific changes in protein expression associated with age

Of the specific age-related protein changes there were a number of novel changes and some points of convergence with previous reports. As a positive control for the animal treatment and proteomic methods, phosphatidylethanolamine binding protein (PEBP), [also known as Hippocampal cholinergic neurostimulating peptide (HCNP)], has been previously demonstrated to decrease with age in the human hippocampus (Yuasa et al., 2001); a result similar to the decrease (46%) we observed. Coronin 1A, an F-actin-binding protein involved in regulation of the cytoskeleton (Galkin et al., 2008), was similarly decreased, and may be associated with alterations in hippocampal structure and decreases in the number of synapses in this brain region (Rutten et al., 2005; Shi et al., 2005). Protein phosphatase 1 (PP1), which has been reported to have a modulatory role in learning and memory (Genoux et al., 2002), was also significantly decreased with age in this study, while the vesicular fusion protein NSF was increased. Expression of peroxiredoxin 6, a potent antioxidant and free radical scavenger found in hippocampal neuroglia (Jin et al., 2005), was increased in old rats, possibly in response to increased oxidative stress that has been reported in aged brain (Donahue et al., 2006; Murali and Panneerselvam, 2007; Squier, 2001) (see (Droge and Schipper, 2007) for review). Interestingly, increased peroxiredoxin expression has been reported in brain with neurodegenerative disorders characterized by oxidative stress. In contrast, glutathione synthetase, which catalyzes the second step in biosynthesis of the antioxidant glutathione, was decreased with aging in this study, providing further evidence for increased oxidative damage in the aged brain (Zhu et al., 2006). It is important to note that these changes with age are evident in comparisons of Young with either Old Intact or Old Impaired animals. Additional changes were observed when the Old Intact and Old Impaired animals were compared.

Specific changes associated with cognitive state

Several expression changes in the hippocampal proteome specific to age-related cognitive decline were not identified in generalized age group comparisons. For example, puromycin-sensitive aminopeptidase (PSA), a proteolytic enzyme essential for hydrolysis of proteins containing polyglutamine sequences (Bhutani et al., 2007), was increased in Old Impaired rats compared to Old Intact rats. This effect was not observed when comparing all Old animals (Intact and Impaired) with Young rats. Although this protein was originally isolated from brain (Hersh and McKelvy, 1981), little is known about its role in neuronal function. Increased PSA expression in hippocampi of cognitively impaired animals may represent a compensatory response to protein misfolding or aggregation in an attempt to degrade accumulating peptides. Likewise, significant increases in the molecular chaperone heat shock 70 family members, heat

shock protein 4 and heat shock protein 105/110 kDa, emerged only when a cognitive state-specific comparison (Old Intact vs. Old Impaired) was made. These chaperones function to ensure proper folding of newly synthesized proteins and target old proteins to degradation machinery to prevent protein aggregation. Heat shock proteins are induced by protein misfolding and oxidative stress, and have been demonstrated to increase in both aged rat brain (Unno et al., 2000; Calabrese et al., 2003; Calabrese et al., 2004) and human hippocampus with Alzheimer's disease (Sultana et al., 2007). This is the first demonstration of increased heat shock protein expression specific to age-related cognitive decline, however, and supports a role of increased stress and/or impaired protein processing.

Alpha enolase was significantly decreased in Old rats compared to Young rats, but was significantly increased in Old Impaired rats compared to Old Intact rats. These data suggest that alpha enolase, an essential glycolytic enzyme (Keller et al., 1994), is differentially regulated by normal aging and the processes underlying age-related cognitive decline. Aldolase, phosphofructokinase, phosphoglycerate mutase and malate dehydrogenase, additional metabolic enzymes, were also increased with cognitive impairment when old impaired animals were compared with all intact animals. This is consistent with published data suggesting that glycolytic enzymes increase in the aged mouse brain, potentially as a compensatory response to decreased metabolic activity (Poon et al., 2006c), and is in further agreement with reports of altered metabolism in age-related cognitive decline (Gage et al., 1984; Rowe et al., 2007; Sonntag et al., 2005). Consistent with these data, aged rats exhibited dysregulation of glucose-utilization pathways in this study, with the majority of protein alterations localized to the ATP-generating steps of glycolysis. This finding is particularly interesting in the context of a recent report of decreased glucose utilization and ATP generation in hippocampi from growth hormone-deficient rats, which serve as a model of brain aging (Sonntag et al., 2006).

Oxidative stress and increased protein oxidation are well-established characteristics of the aged brain and have been implicated in cognitive impairment and hippocampal dysfunction (Miller and O'Callaghan, 2003; Droge and Schipper, 2007; Butterfield et al., 2006). Upregulation of proteins involved in oxidative carbohydrate metabolism in hippocampi of Old, Cognitively Impaired rats may contribute to the generation of reactive oxygen species and free radicals, thereby increasing oxidative stress. Alternatively, oxidative inactivation of carbohydrate metabolism proteins may decrease energy metabolism and contribute to neuronal dysfunction (Butterfield et al., 2006). Human studies demonstrate that cognitive decline is closely related to impaired cerebral blood flow and metabolism, and more recent reports suggest that, while only modest neuronal loss occurs (Gallagher et al., 2003; Rapp and Gallagher, 1996), increased oxidative stress and metabolic derangements likely contribute to broader neuronal dysfunction. (Esiri, 2007; Hoyer, 1982). These more insidious changes also may be related to decreased cerebral blood flow, which renders the aged brain highly susceptible to hypoxic insult (Kietzmann et al., 2001; Ingraham et al., 2008). Alternatively, compromised redox status of these proteins may impair their normal function and enzymatic activity, contributing to decreased glucose utilization and metabolic derangement.

Nearly all conditions favoring ROS production activate hypoxia-inducible factor 1 (HIF-1), which regulates flux through the glycolytic pathway (Seagroves et al., 2001) by inducing expression of metabolic enzymes including pyruvate kinase, aldolase, enolase and lactate dehydrogenase (Schubert, 2005), all of which were upregulated in Old rats compared to Young rats in this study. It has been suggested that the metabolic effects of hypoxia-ischemia are more pronounced in the hippocampus of aged rats, possibly due to the extremely high metabolic demands of this tissue (Siqueira et al., 2004; Macri et al., 2006; Cada et al., 2000; Uecker et al., 2000). It is possible that the combined insults of decreased cerebral blood flow, chronic hypoxia and decreased glucose utilization in aged rats upregulate glycolytic enzymes in

hippocampus as a compensatory response that further contributes to oxidative stress and increased stress proteins, thereby exacerbating cognitive decline in a subset of animals. While the specific roles of these proteins in cognitive decline remain to be determined, further work investigating the biochemical mechanisms of age-related hippocampal dysfunction in rats may help to explain the mechanisms that preserve cognitive function in some aged subjects and impair function in others.

Future studies of proteomic changes with cognitive decline and/or age will require additional technical refinements. While the changes described here are of interest, they represent fairly abundant protein species. Specific strategies to increase sensitivity to detect lower abundance proteins will have to be applied to future studies, such as synaptosomal preparations (Jimenez et al., 2002) or affinity trapping for specific posttranslational modifications (Kruger et al., 2008). Also, while this study concentrated on protein expression, several excellent reports by Butterfield and colleagues demonstrate the importance of examining posttranslational modifications and their effects on protein function in aged hippocampus (Butterfield et al., 2006; Poon et al., 2006b; Poon et al., 2006c; Poon et al., 2006a).

We have described a proteomic analysis of the rat hippocampus in Young and Old animals behaviorally phenotyped for cognitive performance. In agreement with previous findings, a number of alterations including changes in the glycolysis/gluconeogenesis pathway were evident in the comparison between Young and Old animals. When comparing Old cognitively Intact and Old cognitively Impaired animals, many age-related changes were not specifically associated with decreased cognitive performance. Rather, diminished cognitive performance was associated with a distinct set of changes that occurred in addition to age-related changes. The results of our current study provide compelling evidence that cognitive decline is not simply an exacerbation of changes associated with healthy aging, a finding which would not have been apparent without behavioral stratification of animals. These two sets of changes (age-related and cognitive status-specific) within the hippocampal proteome may act synergistically to result in cognitive decline. Extension of these findings across the broader proteome and functional manipulation of specific proteins will enable deciphering of the biomolecular transition from healthy aging to cognitive decline.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

2-DIGE, 2-Dimensional in-Gel Electrophoresis
 5-HT, serotonin
 ASB-14, amidosulfobetaine-14
 BDNF, brain derived neurotrophic factor
 CHAPS, 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
 FDR, false discovery rate
 HCNP, hippocampal cholinergic neurostimulating peptide
 HIF-1, hypoxia-inducible factor 1
 IGF-1, insulin-like growth factor-1
 MALDI-ToF/ToF, matrix assisted laser desorption ionization time-of-flight/ time-of-flight
 MS, mass spectrometry
 NGF, nerve growth factor
 PCA, principal component analysis
 PEBP, phosphatidylethanolamine binding protein
 PP1, protein phosphatase 1

PSA, puromycin-sensitive aminopeptidase
 ROS, reactive oxygen species
 SDS, sodium dodecyl sulfate
 TFA, trifluoroacetic acid.

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Dye	Group (Animal #)	
Cy3	Young Intact (#1)	} Gel 1
Cy5	Old Impaired (#1)	
Cy2	Normalization Pool	
Cy3	Old Intact (#1)	} Gel 2
Cy5	Young Intact (#2)	
Cy2	Normalization Pool	
Cy3	Old Impaired (#2)	} Gel 3
Cy5	Old Intact (#2)	
Cy2	Normalization Pool	
Cy3	Young Intact (#3)	} Gel 4
Cy5	Old Impaired (#3)	
Cy2	Normalization Pool	
Cy3	Old Intact (#3)	} Gel 5
Cy5	Young Intact (#4)	
Cy2	Normalization Pool	
Cy3	Old Impaired (#4)	} Gel 6
Cy5	Old Intact (#4)	
Cy2	Normalization Pool	
Cy3	Young Intact (#5)	} Gel 7
Cy5	Old Impaired (#5)	
Cy2	Normalization Pool	
Cy3	Old Intact (#5)	} Gel 8
Cy5	Normalization Pool	
Cy2	Normalization Pool	

Normalization Pool — Preparative Gel

Figure 1. Experimental Design of 2DIGE Experiment

Hippocampal protein extracts from Young-Intact, Old-Intact, and Old-Impaired (N=5/group) were analyzed by 2-DIGE. Individual animals were randomly assigned to the Cy3 or Cy5 channel to avoid any dye-specific artifacts. The normalization pool group was created by combining 50 μ g of protein from each of the 12 samples used. The normalization pool was run on each gel (50 μ g labeled with Cy2) to normalize quantification across gels. For the preparative gel, 200 μ g of unlabeled normalization pool protein was used.

Figure 2. Behavioral Testing Data

A Latency to reach the escape platform in the acquisition phase of the Morris Water Maze. Animals received one trial/day for 15 days and groups of 3 trials (representing the three possible start locations in the pool) were combined into a training block. Young cognitively intact animals demonstrated marked improvement over the training blocks (lower values indicate better performance). Similar improvement was noted for the old-cognitively intact animals. Latency of the old impaired animals to reach the platform was significantly greater (indicating poorer performance) in block 2 ($p=0.044$) compared to young animals and greater than young or old intact animals during block 3 ($p=0.005$ and $p=0.017$, respectively). **B**, Duration in the Annulus-40 during the Probe trial of the Morris Maze. Young and old intact animals spent

significantly greater time in the Annulus-40 around the platform during the 30 second Probe Trial compared to old-impaired animals (Young Intact vs Old Impaired $p=0.012$ and Old Intact vs Old Impaired $p=0.016$). Points represent individual animals and the horizontal bar represents the group mean.

Figure 3. Example Gel from Study

An image of the preparative (picking) gel with insets showing higher magnifications of specific regions as demonstration of spot density and distribution.

Figure 4. Example Mass Spectrum from Study

An image corresponding to phosphatidylethanolamine binding protein (PEBP), showing the parent MS (top) and the daughter MS/MS of the 1963.95 precursor peptide (bottom). Protein identification was achieved with a MASCOT confidence > 95%.

Figure 5. Set Analysis of Protein Expression Changes

Identified proteins regulated in a statistically significant manner were compared for commonalities and differences. A principal finding is that, for examination of proteomic changes associated with age-related cognitive decline, comparing old and young animals is insufficient and fails to reflect many protein changes specific to cognitive impairment. In this study, comparing only old intact and old impaired animals demonstrated that 9 proteins identified by MS/MS-MS were regulated specifically with cognitive status. Only one of these proteins was detected in the comparison of young and old animals.

Figure 6. Principal Components Analysis of Animal Groups

Using the expression data for the genes regulated with either cognitive performance or with age the relationship of groups was assessed by PCA. The first component related to age and accounted for 70.9% of the study variance. The second component corresponded to cognitive status and accounted for 29.1% of variance.

Figure 7. Glycolysis/Gluconeogenesis pathway changes with age and cognitive decline
Protein expression data were imported into the Ingenuity Pathway Analysis software, which determined multiple enzymes within the glycolysis/gluconeogenesis pathway to be regulated primarily with aging (Old Intact/Young Intact), with additional changes associated with cognitive impairment among old animals (Old Impaired/Old Intact). When both age- and cognitive status-associated changes are examined in combination (Old Impaired/Young Intact), both sets of protein expression changes are evident. Comparisons indicate ratios of protein concentrations in each group.

Table 1

Age-related Changes*

Panther Biological Process	gi number	protein name	Old v Young	Old Intact v Young Intact	Old Impaired v Young Intact
<i>Amino acid metabolism (BP00013)</i>					
	24401	Aspartate aminotransferase, cytoplasmic		↓	
	64192	Quinoid dihydropteridine reductase		↑	
<i>Apoptosis (BP00179)</i>					
	53605	Nucleosome assembly protein 1-like 1	↓		↓
	5500	Protein phosphatase 1, catalytic subunit, beta isoform 1		↓	
<i>Carbohydrate metabolism (BP00001)</i>					
	24189	Aldolase A			↑
	24333	Enolase 1, alpha	↓	↓	↓
	24533	Lactate dehydrogenase A		↑	↑
	65152	Phosphofructokinase, muscle			↑
	60416	Phosphofructokinase, platelet	↑		↑
	18648	Phosphoglycerate mutase 1	↓	↓	↓
	5500	Protein phosphatase 1, catalytic subunit, beta isoform 1		↓	
	25630	Pyruvate kinase, muscle	↑	↑	
	64524	Transketolase		↑	
<i>Cell cycle (BP00203)</i>					
	155151	Coronin, actin binding protein, 1A		↓	
	29474	Coronin, actin-binding protein, 1B	↓		
	53605	Nucleosome assembly protein 1-like 1	↓		↓
<i>Cell proliferation and differentiation (BP00224)</i>					
	155151	Coronin, actin binding protein, 1A		↓	
	29474	Coronin, actin binding protein, 1B	↓		

Panther Biological Process	gi number	protein name	Old v Young	Old Intact v Young Intact	Old Impaired v Young Intact
<i>Cell structure and motility (BP00285)</i>					
	155151	Coronin, actin binding protein, 1A		↓	
	29474	Coronin, actin-binding protein, 1B	↓		
	5500	Protein phosphatase 1, catalytic subunit, beta isoform 1		↓	
	54319	Villin 2			↑
	360950	WD repeat domain 1	↓	↓	↓
<i>Coenzyme and prosthetic group metabolism (BP00081)</i>					
	64192	Quinoid dihydropteridine reductase		↑	
	64524	Transketolase		↑	
<i>Intracellular protein traffic (BP00125)</i>					
	155151	Coronin, actin binding protein, 1A		↓	
	29474	Coronin, actin-binding protein, 1B	↓		
	60355	N-ethylmaleimide sensitive fusion protein	↑	↑	↑
<i>Nucleoside, nucleotide and nucleic acid metabolism (BP00031)</i>					
	53605	Nucleosome assembly protein 1-like 1	↓		↓
	5500	Protein phosphatase 1, catalytic subunit, beta isoform 1		↓	
<i>Other metabolism (BP00289)</i>					
	64040	Aldehyde dehydrogenase family 9, subfamily A1		↑	
	170851	Mitogen activated protein kinase kinase 1			↓
<i>Protein metabolism and modification (BP00060)</i>					
	170851	Mitogen activated protein kinase kinase 1			↓
	29668	Proteasome (prosome, macropain) subunit, alpha type 1		↓	

Panther Biological Process	gi number	protein name	Old v Young	Old Intact v Young Intact	Old Impaired v Young Intact
	29673	Proteasome (prosome, macropain) subunit, alpha type 6	↓		
	5500	Protein phosphatase 1, catalytic subunit, beta isoform 1		↓	
	360593	Protein phosphatase 1E (PP2C domain containing)	↓	↓	
	297593	Ubiquitin specific protease 5	↓		
<i>Sensory perception (BP00182)</i>	117024	Crystallin, mu		↓	
	360950	WD repeat domain 1	↓	↓	↓
<i>Signal transduction (BP00102)</i>	155151	Coronin, actin binding protein, 1A		↓	
	29474	Coronin, actin-binding protein, 1B	↓		↓
	29542	Phosphatidylinositol-binding protein	↓		
	360593	Protein phosphatase 1E (PP2C domain containing)	↓	↓	
<i>Transport (BP00141)</i>	155151	Coronin, actin binding protein, 1A		↓	
	29474	Coronin, actin-binding protein, 1B	↓		
	60355	N-ethylmaleimide sensitive fusion protein	↑	↑	↑
	24825	Transferrin	↓		↑
<i>Unclassified (BP00216)</i>	2133747	Angiotensin I	↑		
	29525	Adenine phosphoribosyl transferase	↑		
	25742757	Glutathione synthetase		↓	
	148747414	Guanine deaminase	↑		
	16758348	Peroxioredoxin 6	↑	↑	
	292072	Angiotensin I		↑	

* Arrows indicate significant up- or down-regulation of protein expression with aging ($p < 0.05$). No arrow indicates that no significant change in protein expression was detected for a particular comparison of animal groups.

Table 2

Cognitive Status-Related Changes*

Panther Biological Process	gi number	protein name	Impaired v Intact	Old Impaired v Old Intact	Old Impaired v Young Intact
<i>Carbohydrate metabolism (BP00001)</i>					
	24189	Aldolase A	↑	↑	↑
	24333	Enolase 1, alpha	↑	↑	↓
	24533	Lactate dehydrogenase A			↑
	24551	Malate dehydrogenase 1, NAD (soluble)	↑	↑	
	65152	Phosphofructokinase, muscle	↑		↑
	60416	Phosphofructokinase, platelet	↑		↑
	18648	Phosphoglycerate mutase 1			
<i>Cell structure and motility (BP00285)</i>					
	54319	Villin 2	↑	↑	↑
	360950	WD repeat domain 1			↓
<i>Protein metabolism and modification (BP00060)</i>					
	291394	CNDP dipeptidase 2 (metallopeptidase M20 family)	↑		↓
	288444	Heat shock 105kDa/110kDa protein 1		↑	
	266759	Heat shock protein 4		↑	
	170851	Mitogen activated protein kinase kinase 1		↑	↓
	50558	Puromycin-sensitive aminopeptidase		↑	
<i>Transport (BP00141)</i>					
	60355	N-ethylmaleimide sensitive fusion protein			↑
	24825	Transferrin	↑		↑
<i>Unclassified (BP00216)</i>					
	51792	Alpha isoform of regulatory subunit A		↑	↑
	53605	Nucleosome assembly protein 1-like 1			↓
	29338	Peroxisomal protein 2	↑		

Panther Biological Process	gi number	protein name	Impaired v Intact	Old Impaired v Old Intact	Old Impaired v Young Intact
	29542	Phosphatidylethanolamine binding protein			↓

* Arrows indicate significant up- or down-regulation of protein expression with cognitive impairment ($p < 0.05$). No arrow indicates that no significant change in protein expression was detected for a particular comparison of animal groups.