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Hippocampal dysregulation of synaptic plasticity-associated proteins with age-related cognitive decline

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Abstract

Age-related cognitive decline occurs without frank neurodegeneration and is the most common cause of memory impairment in aging individuals. With increasing longevity, cognitive deficits, especially in hippocampus-dependent memory processes, are increasing in prevalence. Nevertheless, the neurobiological basis of age-related cognitive decline remains unknown. While concerted efforts have led to the identification of neurobiological changes with aging, few age-related alterations have been definitively correlated to behavioral measures of cognitive decline. In this work, adult (12 Months) and aged (28 months) rats were categorized by Morris water maze performance as Adult cognitively Intact, Aged cognitively Intact or Aged cognitively Impaired, and protein expression was examined in hippocampal synaptosome preparations. Previously described differences in synaptic expression of neurotransmission-associated proteins (Dnm1, Hpca, Stx1, Syn1, Syn2, Syp, SNAP25, VAMP2 and 14-3-3 eta, gamma, and zeta) were confirmed between Adult and Aged rats, with no further dysregulation associated with cognitive impairment. Proteins related to synaptic structural stability (MAP2, drebrin, Nogo-A) and activity-dependent signaling (PSD-95, 14-3-3θ, CaMKIIα) were up- and down-regulated, respectively, with cognitive impairment but were not altered with increasing age. Localization of MAP2, PSD-95, and CaMKIIα demonstrated protein expression alterations throughout the hippocampus. The altered expression of activity- and structural stability-associated proteins suggests that impaired synaptic plasticity is a distinct phenomenon that occurs with age-related cognitive decline, and demonstrates that cognitive decline is not simply an exacerbation of the aging phenotype.

Keywords

Nogo; CamkII; aging; hippocampus; synapse; learning and memory

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Introduction

Age-related cognitive decline impacts a variety of brain functions, and reduces quality of life for aging individuals by diminishing healthspan and increasing dependence. Currently, an estimated 40% of the otherwise healthy population over age 60 is affected by cognitive decline (Small, 2002). Common age-related conditions such as hypertension and heart disease are risk factors for cognitive decline, and are associated with increased severity of cognitive deficits with advancing age (Qiu et al., 2005; Dahle et al., 2009; Okonkwo et al., 2010). Due to lifespan increases, demographic shifts, and health care advances, the percentage of the population over age 60 is expected to increase to 20% by 2050 (Shrestha, 2006). This prevalence of aged individuals is unique in human history, and the incidence of age-related health conditions is expected to rise concomitantly with our increasing lifespan. As such, concerted efforts are needed to understand, prevent, and treat age-related cognitive decline.

Previous characterizations of the hippocampal proteome and transcriptome with aging and cognitive decline (Poon et al., 2006; Blalock et al., 2005; Rowe et al., 2007; Blalock et al., 2003; Butterfield et al., 2006; Freeman et al., 2009b) have identified alterations in neurobiologically-relevant processes associated with advancing age, including increased oxidative stress, decreased glucose utilization and bioenergetic metabolism, and aberrant protein synthesis and trafficking. Although these processes are important to healthy neuronal function, a more immediate cause of cognitive decline is likely dysregulation of neurotransmission and synaptic plasticity. Electrophysiological correlates of hippocampal function are disrupted with aging and learning impairments, and are consistent with unstable encoding of spatial representations (Barnes et al., 1997; Kumar et al., 2007; Norris et al., 1996; Rosenzweig and Barnes, 2003; Wilson et al., 2003). This instability manifests in resistance to LTP induction, facilitation of LTD, and aberrant spatiotemporal activation of ensemble networks. These characteristics may be related to atypical synapse morphology, neurotransmitter synthesis and receptor signaling, and neuronal gene and protein expression (Liu et al., 2008; Shi et al., 2005; Burke and Barnes, 2006). Additional work is required, however, to establish a definitive link between these phenomena and impaired cognitive function.

Using a rodent model of age-related cognitive decline that enables segregation of aged rats into cognitively intact and impaired groups based on Morris water maze performance, we have demonstrated two distinct shifts in the hippocampal cytosolic proteome with age-related cognitive decline: one related to aging and a second specific to cognitive function (Freeman et al., 2009b). Further, we recently reported age-related dysregulation of components of the hippocampal synaptoproteome with roles in initiation and modulation of neurotransmission (VanGuilder et al., 2010). The goal of the current study was to identify synaptic proteins regulated specifically with age-related cognitive decline and to differentiate this phenomenon from the general effects of aging. This investigation focused on proteins that mediate activity-responsive synaptic signaling and structural remodeling, and that have well-characterized regulatory functions in hippocampus-dependent spatial learning and memory (Elgersma et al., 2004; Khuchua et al., 2003; Kojima and Shirao, 2007; Migaud et al., 1998; Miller et al., 2002; Mizui et al., 2005; Zagrebelsky et al., 2010). We observed increased expression of proteins vital to synaptic structural stability (MAP2, drebrin, Nogo-A) and decreased expression of synaptic activity-dependent signaling proteins (PSD-95, 14-3-3θ, CaMKIIα) that strongly correlate with declining cognitive performance. These findings provide evidence for disrupted hippocampal synaptic plasticity specifically in cognitively impaired aged animals which may act synergistically with age-related decrements of neurotransmission efficacy.

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Materials and Methods

Animals

Three independent cohorts of male Fischer 344 x Brown Norway (F1) hybrid rats (see Table 1 for cohort information) were obtained from the National Institute on Aging colony at Harlan Industries (Indianapolis, IN). Synaptosome samples used in aging-only analyses (set 1) were derived from Young-adult, Adult, and Aged rats (VanGuilder et al., 2010). Rats were singly housed in laminar flow cages (Polysulfone) in the OUHSC specific pathogen-free Barrier Facility with water and food freely available (Purina Mills, Richmond, IN). Environmental controls maintained a 12-hour light/dark cycle and constant temperature and humidity. One week following completion of behavioral testing, rats were sacrificed by decapitation without anesthesia, and the hippocampi rapidly dissected for preparation of synaptosomes (set 2). Alternatively, rats were perfusion-fixed, and their brains collected for immunohistochemical analysis (set 3). At necropsy, animals were examined for exclusionary criteria including peripheral tumors, frank kidney disease and cardiac hypertrophy as well as gross brain neuropathology (e.g., cortical atrophy and pituitary tumors). The OUHSC animal facilities are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, and all animal procedures were approved by the Institutional Animal Care and Use Committee in compliance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals and the National Research Council's Guide for the Care and Use of Laboratory Animals.

Morris water maze testing

Rats were acclimatized to the OUHSC Barrier Facility for two weeks prior to cognitive assessment conducted with a variation of the Morris water maze using methods similar to those previously described (Freeman et al., 2009b; Mitschelen et al., 2009). The water maze consisted of a galvanized metal tank 1.7m in diameter and 0.6m in height. Water made opaque with non-toxic water-based white food coloring was added to a depth of 25cm, and a retractable 12cm escape platform was fixed 2cm below the water's surface. A curtain with fixed-position visual cues, serving as reference cues for the location of the escape platform, surrounded the maze pool. A center-mounted camera located 1.6m above the water's surface provided image input to an automated tracking system (Noldus Ethovision XT, Wageningen, Netherlands) that recorded water maze performance. Task acquisition was conducted in four training blocks consisting of five individual trials performed over two days, for a total of eight days of task acquisition with the escape platform located in the same position across all days of training. Rats were placed into the maze, facing the wall of the pool in one of four locations with start positions pseudo-randomized, and were given 60s to locate the escape platform based on surrounding spatial cues. Path length to find the platform was the dependent measure, with shorter path lengths indicating better performance. After completion of each acquisition block (i.e., on days 2, 4, 6 and 8), a probe trial was performed with the escape platform removed. Rats were placed into the maze and the mean proximity to the platform location, duration in the annulus-40 (the area 40cm around the platform location), cumulative distance, and mean swim velocity were recorded during the first 30 seconds. To avoid extinguishing memory of the platform location, the platform was then replaced and rats were given an additional 60s to locate it using the surrounding cues. Probe trial data were used to segregate Aged animals into cognitively Intact and Impaired groups relative to Adult group performance, allowing retrospective analysis of acquisition phase data by group. Mean proximity to the escape platform location was used as the primary measure of cognitive performance on probe trials based on demonstration of its superior sensitivity compared to alternative measures (Maei et al., 2009). The number of cumulative platform location crossings was used as a secondary measure of cognitive performance (Terry, 2009). To ascertain successful task acquisition, data were statistically

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analyzed across blocks by one-way repeated measures ANOVA with Holm-Sidak post hoc testing. Significance of group differences for individual acquisition blocks and probe trials was assessed by one-way ANOVA with Student Newman Keuls post hoc testing. Two days following conclusion of water maze testing, visual performance was assessed over four consecutive swim trials using a visible platform. One week later, animals were sacrificed and brains dissected for hippocampal synaptosome isolation or immunohistochemical analysis.

Hippocampal Synaptosome Preparation

Hippocampal synaptosomes were prepared as previously described (VanGuilder et al., 2010). Immediately following sacrifice, individual hippocampi from each rat were rapidly dissected into ice-cold buffered sucrose (320mM sucrose, 3mM HEPES, 1mM Na_3VO_4, pH 7.4) and incubated on ice for 30min with buffer replaced twice at 10min intervals. Hippocampi were homogenized in 8mL buffered sucrose with a mechanically-driven dounce homogenizer and separated by differential centrifugation. Whole homogenates were centrifuged to pellet nuclear/cytoskeletal fractions (12min, 1000 × g, 4°C). The resulting supernatants were then centrifuged to pellet the synaptosomal fractions (16min, 25,000 × g, 4°C). Synaptosomal fractions were washed by resuspending in 5mL buffered sucrose and centrifuging to re-pellet samples (16min, 25,000 × g, 4°C). Synaptosome samples were then resuspended in a detergent-based protein lysis buffer containing protease and phosphatase inhibitors (100mM NaCl, 20mM HEPES, 1mM EDTA, 1mM dithiothreitol, 1.0% Tween20, 1mM Na_3VO_4, 1 Complete Mini EDTA-Free Protease Inhibitor Cocktail Tablet (Roche Applied Science, Indianapolis, IN) for every 10 mL lysis buffer), and soluble protein extracted by sonication on ice. Synaptosome lysates were incubated for 15min at 4°C with gentle rocking, insoluble protein was pelleted by centrifugation (12min, 10,000 × g, 4°C), and the soluble protein-containing supernatant was stored at −80°C for subsequent experimentation.

Immunoblotting

Sample protein yields were determined by bicinchoninic acid quantitation (Pierce, Rockford, IL) and adjusted to a concentration of 2μg/μL in protein lysis buffer and LDS sample buffer (Invitrogen, Carlsbad, CA). 10μg of each prepared protein sample was denatured by heating to 95°C for 5min prior to separation by SDS-PAGE using Criterion Tris-HCl precast gels (4–20% acrylamide gradient, 1mm thick, 26 wells; BioRad, Hercules, CA, USA). To ensure equal protein content, one gel containing all study samples was fixed with 10% ethanol/1% citric acid and stained with Deep Purple total protein stain according to manufacturer's instructions (GE LifeSciences) as previously described (VanGuilder et al., 2010). For immunoblotting, SDS-PAGE separated proteins were transferred to PVDF membranes (HyBond, GE Healthcare) and blocked with 5% nonfat milk in PBS with 1.0% Tween-20 (PBST) prior to overnight incubation with primary antibodies (Table 2) at 4°C. Membranes were washed with PBST, incubated with horseradish peroxidase-conjugated, species-appropriate secondary antibodies, and developed with enhanced chemiluminescence substrate (GE Healthcare). Immunoreactive bands were imaged on film, digitized at a resolution of 800dpi, and quantitated using automated digital densitometry software (ImageQuant TL, Molecular Dynamics, Sunnyvale, CA, USA). Immunoblot data were normalized to corresponding whole-lane densitometric volumes of the total protein stained gel. Pairwise comparisons (i.e., Adult/Aged and Aged Intact/Aged Impaired) were assessed by unpaired two-tailed t-test. Analysis of three groups (i.e., Young/Adult/Aged and Adult/Aged Intact/Aged Impaired) was performed by one-way ANOVA with Student Newman Keuls post hoc tests.

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Quantitative RT-PCR

Hippocampal synaptosome samples were homogenized in 300μL TriReagent (Molecular Research Center, Cincinnati, OH) by bead mill (Retsch TissueLyzer II, Qiagen, Valencia CA, USA) as previously described (Rountree et al., 2010; Freeman et al., 2009a). RNA was isolated from synaptosomal homogenates by addition of 10% BCP and standard phase separation, followed by overnight precipitation with isopropanol at -20°C. RNA was purified using the Qiagen RNeasy Mini kit (Qiagen), and quality and quantity were assessed by microfluidics chip (Agilent 2100 Expert Bioanalyzer Nano Chip, Agilent, Palo Alto, CA) and spectrometry (NanoDrop ND1000; Thermo Scientific, Wilmington, DE), respectively. Only samples with an RNA integrity number >8 were included in subsequent analyses.

cDNA was synthesized from purified RNA with the ABI High capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). For each sample, 1μg RNA was reacted with random primers, dNTPs, and MultiScribe Reverse Transcriptase enzyme using a GeneAmp PCR 7300 System (Applied Biosystems), as previously described (Brucklacher et al., 2008; Freeman et al., 2010; Freeman et al., 2009a). qPCR analysis of targets of interest was performed using standard laboratory methods, with 384-well optical plates, TaqMan Assay-On-Demand (Applied Biosystems, Foster City, CA, USA) gene-specific primers/probe assays and a 7900HT Sequence Detection System (Applied Biosystems) (Brucklacher et al., 2008; Freeman et al., 2010). Gene expression assays included Mtap2 (MAP2, Rn00565046_m1), Dbn1 (drebrin, Rn00578869_m1), Rtn4 (Nogo-A, Rn00582903_m1), Dlgh4 (PSD-95, Rn00571479_m1), Ywhaq (14-3-3θ, Rn00820722_g1) and Camk2 (CaMKIIα, Rn00563883_m1). Relative gene expression was calculated with SDS 2.2.2 software using the $2^{-\Delta\Delta Ct}$ analysis method with β-actin as an endogenous control. Statistical analysis was performed by one-way ANOVA, with Student Newman Keuls post hoc tests if ANOVA $p<0.05$.

Immunohistochemistry

One week following completion of cognitive assessment, rats used for immunohistochemical localization of protein targets were euthanized. Animals were anesthetized with ketamine/xylazine and transcardially perfused with 6U/mL heparin (sodium salt) in PBS followed by phosphate-buffered 4% paraformaldehyde (pH 7.4). Brains were extracted and hemisected sagittally, immersion-fixed in 4% paraformaldehyde (pH 7.4) overnight at 4°C, rinsed twice in PBS, and impregnated with 30% sucrose. Tissue samples were embedded in Tissue-Tek optimal cutting temperature compound (Sakura Finetek, Torrance, CA, USA), frozen in isopentane on dry ice, and stored at −80°C. Four Aged Intact and four Aged Impaired rats, with three replicate sections per rat per antibody, were included in this analysis. Hippocampi were cryosectioned in the sagittal plane (HN 505E, Microm International, Walldorf, Germany) at −22°C. Ten-micron sections were postfixed with 2.0% paraformaldehyde, pH 7.4, for 10min at room temperature and blocked with 10% donkey serum (Jackson ImmunoResearch, WestGrove, PA, USA) in PBS/0.1% Triton X-100 at room temperature for one hour. Sections were then incubated with primary antibodies (Table 2) in blocking solution overnight at 4°C, washed with PBS/0.1% Triton X-100, and visualized by incubation for one hour at room temperature with affinity-purified fluorescence-conjugated donkey anti-rabbit secondary antibody (DyLight 649, Jackson ImmunoResearch) diluted 1:1000 in blocking solution. Sections were counterstained with Hoechst 33258 (5μg/mL, Invitrogen, Carlsbad, CA), added for the last 10 minutes of incubation with secondary antibody. After washing, sections were coverslipped with Aqua Poly/mount (Polysciences, Warrington, PA, USA) for imaging by confocal microscopy. All images were acquired using a confocal laser scanning microscope (Leica TCS SP2 AOBS, Exton, PA, USA) equipped with a UV-diode laser for Hoechst (405nm) and a helium-neon laser for DyLight 649 (633nm). Hippocampal subregions (CA1, CA3, DG) of Aged Intact

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and Aged Impaired rats were imaged as 8μm series of 16 optical sections (0.5μm step size, 512×512 pixel resolution) using identical laser settings optimized for each target protein and presented as maximum projections of z-stacks. For each target protein and hippocampal subregion, noise reduction was performed using identical linear contrast/brightness adjustments with Adobe Photoshop CS4 software (Adobe Systems, San Jose, CA, USA).

Results

Behavioral characterization

To assess cognitive status, Adult (12 months, n=5) and Aged rats (28 months, n=15) were tested using a variation of the Morris water maze consisting of four acquisition (training) blocks and four interpolated probe (test) trials. Classification of rats by cognitive performance was performed using individual rats' mean performance across all probe trials. After completion of each acquisition block, the escape platform was removed and a probe trial was conducted to assess animals' ability to retain the platform location based on the surrounding contextual cues. Search accuracy was measured by mean proximity to the platform location, with lower values indicating better performance (Maei et al., 2009). Aged rats performing within the range of the Adult group were classified as Aged Intact (n=8), while those that performed worse than the Adult range (ie., higher mean proximity) were classified as Aged Impaired (n=7) (Figure 1A). Aged Impaired rats maintained a mean proximity to the escape platform location that was significantly further from the target area (68±1.6cm) than both Adult (48±1.9cm) and Aged Intact (52±2.4cm) groups ($p<0.001$, Student Newman Keuls post hoc tests). As expected, there was no difference in probe trial performance between Adult and Aged Intact rats. Performance-based segregation was evident in all individual probe trials (Figure 1B). Adult rats performed consistently well across probe trials, and no statistical differences were evident between the performance of Adult and Aged Intact groups. Aged Impaired rats performed significantly worse (i.e., maintained significantly higher proximity-to-platform measures) than Adult rats on all four probe trials ($p<0.05$ to $p<0.001$, Student Newman Keuls post hoc tests), and significantly worse than Aged Intact rats on probe trials 2, 3 and 4 ($p<0.001$, Student Newman Keuls post hoc tests). Similar results were obtained using measurements of rats' cumulative number of platform crossings during probe trials ($p<0.001$). Aged Impaired rats crossed the escape platform location significantly fewer times (6.4±0.97 crossings) over the course of probe trials than either Aged Intact (12.3±.1.0 crossings) or Adult (14.8±1.36 crossings) rats ($p<0.001$, Student Newman Keuls post hoc tests). Using these classifications retrospective analysis of performance by age- and cognition-based groupings throughout acquisition was performed. During task acquisition, rats developed a visual cue-based, spatially focused search pattern for the fixed-location escape platform, as indicated by decreasing search path length (Figure 1C). The entire cohort of rats improved in performance across acquisition blocks, as assessed by one-way repeated measures ANOVA ($p<0.001$). Further, repeated measures ANOVA analysis of individual age and cognition groups revealed significantly decreased path length as acquisition progressed ($p<0.001$), demonstrating that all groups successfully acquired the spatial task. There was no statistical difference in acquisition performance between Adult and Aged Intact rats on any training block. Aged Impaired rats demonstrated less improvement with training and performed significantly worse than other groups on blocks 3 and 4 ($p<0.001$) as determined by Student Newman Keuls post hoc tests. Importantly, these differences were not due to confounding physical deficits, as there were no group differences in path length to a visible platform, mean swim velocity, or total distance swum during assessment of physical capabilities (data not shown). Further, body mass measurements of Aged rats were not different between Intact and Impaired groups (data not shown). Only rats determined to be free of gross pathology (e.g., enlarged kidneys, peripheral tumors, cardiovascular hypertrophy) and frank neuropathology (e.g., pituitary

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tumors, lateral ventricle enlargement, severe cortical atrophy) at necropsy were included in behavioral data analysis and downstream molecular analyses.

Age-related differences in expression of neurotransmission-regulating proteins

We have previously reported decreased synaptosomal expression of a number of synaptic proteins between adulthood and advancing age [animal set 1, (VanGuilder et al., 2010)]. To confirm these findings in an independent animal experiment, expression of synapsin 1, synaptophysin, SNAP25, VAMP2, and dynamin 1 was quantitated by immunoblotting hippocampal synaptosomes (animal set 2, Adult vs. Aged comparison) without consideration of their cognitive status (Figure 2A). In Aged rats, expression of these proteins was again found to be significantly decreased by 20% to 30%, confirming our previous observations ($p<0.05$ to $p<0.01$, unpaired two-tailed t-test). To determine whether these age-regulated proteins are differentially expressed with cognitive impairment, Aged rats were segregated by cognitive performance into Intact and Impaired groups. When immunoblot data were analyzed according to cognitive performance-based groupings, no differences in synapsin 1, synaptophysin, SNAP25, VAMP2 or dynamin expression were observed (animal set 2; Aged Intact vs. Aged Impaired comparison, unpaired two-tailed t-test), demonstrating that these age-regulated decreases in protein expression are not further reduced with cognitive decline (Figure 2B). Similarly, syntaxin 1, synapsin 2, hippocalcin, 14-3-3ζ, 14-3-3ε, and 14-3-3γ, which were previously identified as proteins differentially expressed between Young-Adult and Adult/Aged rats (VanGuilder et al., 2010), were also unaffected by cognitive status in Aged animals (Figure 2C).

Correlations between cognitive performance and expression of synaptic plasticity-associated proteins

To test the hypothesis that cognitive decline is associated with dysregulation of molecular correlates of hippocampal synaptic plasticity, structural stability-associated proteins (MAP2, drebrin, Nogo-A; Figure 3) and synaptic activity-related proteins (PSD-95, 14-3-3θ, CaMKIIα; Figure 4) were quantitated by immunoblotting hippocampal synaptosomes prepared from behaviorally characterized rats (animal set 2). Protein expression measurements for individual animals were tested for correlation with Morris water maze probe trial mean proximity-to-platform values indicative of cognitive performance (i.e., higher mean proximity values indicate poorer performance). Compared to Aged Intact rats, MAP2 content was significantly elevated in Aged Impaired rats (177±11% of Intact, $p<0.01$, unpaired two-tailed t-test). Individual animal MAP2 expression was positively correlated with mean proximity to platform ($r=0.72$, $p<0.01$, Pearson correlation), demonstrating an inverse relationship between hippocampal MAP2 content and cognitive performance. Similarly, expression of drebrin was significantly increased in Aged Impaired rats compared to their age-matched, cognitively Intact counterparts (133±9% of Intact, $p<0.01$, unpaired two-tailed t-test), and was positively correlated with mean proximity to the escape platform ($r=0.81$, $p<0.001$, Pearson correlation). Nogo-A expression was also significantly increased in cognitively impaired rats (187±27% of Intact, $p<0.05$, unpaired two-tailed t-test) and positively correlated with mean proximity to the escape platform ($r=0.70$, $p<0.01$, Pearson correlation). These three structural correlates of synaptic plasticity proteins were significantly upregulated with cognitive decline, and higher synaptosomal expression was statistically correlated with poorer cognitive function in individual animals.

Examination of synaptic activity-related targets demonstrated a direct relationship between cognitive performance and protein expression (Figure 4). PSD-95 expression was significantly decreased in hippocampal synaptosomes of Aged Impaired rats compared to Aged Intact rats (66±12% of Intact, $p<0.05$, unpaired two-tailed t-test) and negatively correlated with mean proximity to the escape platform ($r=-0.63$, $p<0.05$, Pearson

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correlation). 14-3-3θ was also significantly downregulated in Aged Impaired rats (78±7% of Intact, $p<0.01$, unpaired two-tailed t-test) and was negatively correlated with mean proximity to the escape platform ($r=-0.54$, $p<0.05$, Pearson correlation). Finally, CaMKIIα content was significantly lower in Aged Impaired rats (80±4% of Intact, $p<0.05$, unpaired two-tailed t-test) and negatively correlated with mean proximity to the escape platform ($r=-0.59$, $p<0.05$, Pearson correlation). These data suggest a direct relationship between synaptosomal levels of these proteins and cognitive performance, in which lower expression is statistically associated with poorer cognitive function.

Synaptic expression of cognition-associated proteins is not regulated with advanced aging

To determine whether plasticity-related proteins regulated with cognitive status are also regulated with increasing age, MAP2, drebrin, Nogo-A, PSD-95, 14-3-3θ and CaMKIIα expression was assessed by immunoblotting hippocampal synaptosomes from two cohorts of Adult and Aged rats regardless of cognitive status (sets 1 and 2; Figure 5). As previously reported, PSD-95 and 14-3-3θ are expressed at significantly lower levels in Adult and Aged rats than in Young rats (VanGuilder et al., 2010). No additional age-related alterations in protein expression were detected, however, between Young, Adult and Aged rats. Notably, these proteins were stably expressed between Adult and Aged groups, and expression levels were comparable between the two independent animal cohorts.

Evaluation of synaptic mRNA expression

Potential regulation of plasticity-associated protein expression by local mRNA content was evaluated by qPCR analysis of synaptosomes prepared from Aged Intact and Aged Impaired rats. No differences in expression of MAP2, drebrin, PSD-95, 14-3-3θ, of CaMKIIα transcripts were detected between groups (Figure 6). The Nogo-A transcript was not expressed at detectable levels. This is likely due to the fact that most Nogo-A transcript is expressed in oligodendrocyte cell bodies and neuronal somata, and is therefore not present in synaptosomes (Chen et al., 2000; GrandPre et al., 2000; Huber et al., 2002).

Immunohistochemical localization of cognition-associated protein targets

To enable localization of protein targets of interest to key hippocampal subregions, a second cohort of Adult and Aged rats was behaviorally-characterized (animal set 3). Adult (13 months, n=9) and Aged (26 months, n=15) rats were assessed for cognitive performance in the Morris water maze as described above. Animal groups were segregated according to probe trial performance, indicated by mean proximity to the platform location (Figure 7A). In agreement with our previous findings, Aged rats demonstrated characteristic stratification by cognitive function, with one subset (Aged Intact, n=10) performing within the range of Adults and others performing worse than the Adult range (Aged Impaired, n=5). Aged Impaired rats maintained a distance of 65±2.5cm from the center of the escape platform location, which was significantly further ($p<0.001$, Student Newman Keuls post hoc tests) than both Adult (46±3.8cm) and Aged Intact (47±3.4cm) groups. This segregation was apparent on all four individual probe trials (Figure 7B). Adult and Aged Intact groups performed similarly across probe trials, while Aged Impaired rats maintained a significantly higher mean proximity to the platform location (trials 1, 3 and 4: $p<0.001$ vs. Adult and $p<0.001$ vs. Aged Intact; trial 2: $p<0.05$ vs. Adult and $p<0.05$ vs. Aged Intact; Student Newman Keuls post hoc tests). Retrospective analysis of acquisition data demonstrated that all animals acquired the spatial task as training progressed (one-way repeated measures ANOVA, $p<0.001$). Similar results were obtained when the acquisition performance of segregated groups (i.e., Adult, Aged Intact, Aged Impaired) was assessed. Adult and Aged Intact rats demonstrated a similar degree of marked improvement throughout acquisition, indicated by decreasing path length to locate the escape platform ($p<0.001$, one-way

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repeated measures ANOVA). Although Aged Impaired rats also acquired the task ($p=0.029$, one-way repeated measures ANOVA), they performed consistently worse than Adult and Aged Intact rats as determined by one-way ANOVA with Student Newman Keuls post hoc tests (block 2: $p<0.05$, block 3: $p<0.001$, block 4: $p<0.001$).

Given the well-described neuronal functions and distributions of MAP2, CaMKIIα, and PSD-95, which were differentially expressed between Aged Intact and Aged Impaired rats and significantly correlated with cognitive performance in this work, these proteins were selected for immunohistochemical localization studies (n=4/group). MAP2 exhibited a characteristic pattern of immunoreactivity, delineating dense networks of dendrites in CA1, DG and CA3. Of note is the increased MAP2 immunoreactivity in Aged Impaired rats in all three hippocampal subregions (Figure 8, left). Interestingly, MAP2+ dendrites in CA1 of Aged Impaired rats had a tortuous appearance suggestive of aberrant outgrowth that was not observed in other subregions. CaMKIIα immunoreactivity was largely distributed around neuronal cell bodies, with punctate staining apparent in synaptic compartments, and appeared to decrease uniformly throughout CA1, DG and CA3 with cognitive impairment (Figure 8, center). PSD-95 immunoreactivity identified (Figure 8, right) well-defined postsynaptic densities apparent along neuronal projections throughout CA1, DG and CA3. In addition to a generalized decrease in PSD-95 intensity in Aged Impaired rats compared the Aged Intact group, PSD-95+ puncta appeared to decrease in number throughout the hippocampus, indicating a potential loss of postsynaptic terminals. In total, altered immunoreactivity for these three plasticity-related proteins was apparent to varying degrees in all three hippocampal subregions examined.

Discussion

Advancing age is a primary risk factor for cognitive decline, including impaired hippocampus-dependent spatial learning and memory. The occurrence of age-related cognitive decline is heterogeneous in the human population and in animal models of aging, which suggests that age-related neurobiological changes alone are not sufficient to cause cognitive decline. In this study, the synaptic expression of proteins demonstrated to be functionally necessary for hippocampal plasticity and spatial learning and memory was evaluated in a rodent model of aging and cognitive decline. Our current studies demonstrate novel and specific dysregulation of synaptic plasticity-associated proteins with cognitive impairment in Aged rodents that correlates with Morris water maze performance. These protein expression changes occur in addition to the age-related alteration of neurotransmission-regulating protein expression (VanGuilder et al., 2010) which is not associated with cognitive performance. Cognitive decline-specific expression changes identified include upregulation of synaptic proteins that mediate structural stability and remodeling (MAP2, drebrin, Nogo-A) and downregulation of synaptic activity-responsive proteins (PSD-95, 14-3-3θ, CaMKIIα). Altered expression of these proteins is evident throughout the hippocampus, and is not regulated at the level of synaptic transcript content.

Aberrant hippocampal synaptic plasticity, in the form of impaired electrophysiology and synaptic remodeling, has been reported in numerous studies modeling human aging and cognitive decline (Boric et al., 2008; Casadesus et al., 2004; O'Callaghan et al., 2009; Platano et al., 2008; Ramsey et al., 2004; Tombaugh et al., 2002; Wang et al., 2006). Examination of cognitive decline and expression of proteins that mediate mechanisms of synaptic plasticity, however, has been lacking. We have identified significant, cognitive decline-specific decreases in hippocampal expression of three activity-responsive proteins (PSD-95, 14-3-3θ, CaMKIIα) with extensive roles in synaptic plasticity. PSD-95 (postsynaptic scaffolding protein 95kDa) recruits and clusters NMDA and AMPA receptors, ion channels, cytoskeletal components, and signal transduction molecules in response to

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synaptic activity (Beique et al., 2006; Kim et al., 1995; Vickers et al., 2006). PSD-95 also contributes to the maturation of presynaptic terminals and dendritic spines and the stabilization of established synapses (El-Husseini et al., 2000; Okabe et al., 2001). Transgenic deletion of PSD-95 facilitates hippocampal LTP but also causes severe spatial learning and memory impairment (Carlisle et al., 2008; Migaud et al., 1998). Alternatively, overexpression of PSD-95 increases the frequency and amplitude of miniature excitatory postsynaptic currents and converts silent synapses into functional signaling units (Stein et al., 2003). During spatial learning and memory formation, PSD-95 is recruited to synaptic lipid rafts, suggesting that regulated localization as well as expression of this protein is a necessary component of synaptic plasticity (Delint-Ramirez et al., 2008). CaMKIIα, which acts both pre- and postsynaptically, also has well-defined roles in neurotransmission and synaptic plasticity (Lu and Hawkins, 2006; Wang, 2008). The presynaptic actions of CaMKIIα include modulation of vesicle exocytosis rates, which is executed in part through depolarization-induced activation, mobilization to synaptic vesicle clusters, and phosphorylation of synapsin I and VAMP2 (Chi et al., 2001; Nielander et al., 1995; Tao-Cheng et al., 2006). Postsynaptically, CaMKIIα regulates NMDA receptor expression and localization and AMPA receptor insertion into the postsynaptic membrane (Lu and Hawkins, 2006; Park et al., 2008). Further, CaMKIIα promotes synaptic formation, strengthening, and integration into existing neural circuits (Asrican et al., 2007; Matsuo et al., 2009; Miller et al., 2002; Zha et al., 2009). Like PSD-95, loss of CaMKIIα activity results in severe electrophysiological abnormalities associated with impaired synaptic plasticity and memory formation, while overexpression of CaMKIIα improves cognitive performance as assessed by Morris water maze testing (Elgersma et al., 2004; Giese et al., 1998; Miller et al., 2002; Poulsen et al., 2007; Silva et al., 1992).

The roles of the signal transduction-modulating protein 14-3-3θ (YWHAQ) in synaptic plasticity include modification of synaptic morphology and signaling. 14-3-3 proteins regulate the distribution and activity of large networks of proteins including tryptophan hydroxylase, tyrosine hydroxylase, synaptopodin 2, and kinesin (Bi et al., 1997; Faul et al., 2005; Ichimura et al., 1987; Yamauchi et al., 1981). 14-3-3θ expression is induced with localization pathway-mediated learning, and has been reported to increase in aged rats without cognitive impairment after Morris water maze training (Rowe et al., 2007; Swofford and DeBello, 2007). Through activity-dependent interactions with numerous binding partners including protein kinase C, 14-3-3θ has many potential roles in synaptic plasticity and neuronal function (Skoulakis and Davis, 1998). Decreased synaptic expression of PSD-95, CaMKIIα and 14-3-3θ specifically with cognitive impairment in Aged rats indicates a loss of activity-responsive modulation of synaptic efficacy that likely interferes with learning and memory formation processes. The demonstrated impairment of cognitive performance with genetic deletion or mutation of these proteins in previous reports suggests that restoring normal expression of these proteins may prevent or reverse age-related cognitive decline.

Synaptic activity-responsive structural remodeling is a dynamic process necessary for both synaptogenesis and modification of existing synapses. Specifically in Aged cognitively Impaired rats, hippocampal expression of proteins (MAP2, drebrin, Nogo-A) that may contribute to synaptic rigidity by inhibiting synaptic remodeling is increased and correlates with the degree of cognitive impairment. MAP2 (microtubule-associated protein 2) promotes microtubule assembly and stability by crosslinking microtubule networks in dendritic compartments (De Camilli P. et al., 1984; Dinsmore and Solomon, 1991; Matus et al., 1981). Targeted disruption of MAP2 decreases microtubule bundling, reduces dendritic microtubule density, and impairs dendritic elongation, leading to learning and memory deficits (Harada et al., 2002; Khuchua et al., 2003; Teng et al., 2001). Overexpression of MAP2 inhibits motor activity and disrupts organelle transport (Sheetz et al., 1989; Drewes et

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al., 1998), and may also impede the reorganization of dendritic cytoarchitecture. Immunohistochemical visualization of MAP2 revealed increased expression throughout the hippocampus, as well as a dense, tortuous appearance of MAP2-immunoreactive dendrites that may reflect structural abnormalities stemming from an overabundance of microtubule crosslinking. Drebrin (developmentally-regulated brain protein) is an actin-binding protein enriched in dendritic spines that contributes to synaptic reorganization through modulation of actin dynamics (Ishikawa et al., 1994; Shirao et al., 1992; Shirao et al., 1994). Drebrin mediates synaptic structural reorganization by dissociating actin from tropomyosin, preventing actin filament crosslinking through competitive binding, and exposing actin filaments for cleavage by gelsolin (Hayashi et al., 1996; Ishikawa et al., 1994). Dysregulation of drebrin expression is associated with abnormal dendritic spine morphology, synaptic dysfunction and cognitive deficits in both humans and animal models (Kojima and Shirao, 2007; Mizui et al., 2005). Nogo-A (neurite-outgrowth inhibitor A) is an abundant myelin-associated protein that prevents neurite outgrowth, in part through inhibition of integrin signaling, suppression of LIMK1 activation, and PirB receptor-mediated POSH/Shroom3 complex activation (Dickson et al., 2010; Hu and Strittmatter, 2008). Nogo-A is concentrated in pre- and postsynaptic compartments (Raiker et al., 2010), where it stabilizes synaptic structure (Zagrebelsky et al., 2010). The active Nogo-66 domain of Nogo-A suppresses hippocampal LTP induced via NMDA receptor signaling, while selective loss of the Nogo-66 receptor NgR1 enhances hippocampal LTP, suggesting an acute role in inhibition of synaptic activity as well as of structural remodeling (Lee et al., 2008; Raiker et al., 2010). Nogo-A has emerged as a promising therapeutic target for cognitive deficits due to the beneficial cognitive effects of post-stroke Nogo-A inactivation (Gillani et al., 2010; Lenzlinger et al., 2005), although these effects are not always evident in transgenic deletion models (Marklund et al., 2009). The activity-dependent and structural anti-plastic actions of Nogo-A may be mediated by NgR1 signaling, as loss of NgR1 in adult mice leads to aberrant dendritic spine populations consistent with immature synapses (Lee et al., 2008). Additionally, loss of Nogo-A signaling through neutralizing antibodies, transgenic knockout, and shRNA-knockdown dysregulates hippocampal pyramidal cell dendrite structure, dendritic spine morphology, and axon arborization (Zagrebelsky et al., 2010). These reports demonstrate a stabilizing effect of Nogo-A on hippocampal neurons and suggest that Nogo-A over-expression may lead to inhibition of synaptic plasticity processes consistent with the decreased cognitive performance observed in this study. Together, the upregulated synaptic expression of MAP2, drebrin, and Nogo-A with age-related cognitive decline may have far-reaching inhibitory effects on activity-responsive structural reorganization, leading to a maladaptive rigidity of synaptic structure that could impede the dynamic cytoskeletal modifications underlying the synaptic plasticity necessary for memory formation and retention.

It is interesting to note that previous genomic examinations of aging and cognitive decline have identified age-regulated genes that correlate to cognitive performance (Blalock et al., 2003; Rowe et al., 2007). We have previously reported age-related declines in synaptosomal neurotransmission-regulating protein expression (VanGuilder et al., 2010) as well as age- and cognition-specific alterations in the unfractionated hippocampal proteome (Freeman et al., 2009b). In agreement with the genomic literature, we have also observed metabolic proteins (e.g., aldolase and enolase) (Freeman et al., 2009b) and synaptic proteins (14-3-3θ and PSD-95) that are regulated with both increasing age (VanGuilder et al., 2010) and cognitive decline. The dysregulation of plasticity-associated proteins reported here is specific to aged, cognitively impaired rats, as no general effects of aging between adulthood and advanced age were apparent. The complex interaction of age-regulated and cognition-associated hippocampal molecular changes in the etiology of cognitive decline remains to be fully understood.

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In summary, the findings of this study suggest a molecular basis for impaired hippocampus-dependent learning and memory that involves components of structural synaptic rigidity and attenuated activity-dependent synaptic plasticity. These data are consistent with reports of decreased populations of mature synapses and reduced experience-based synaptic remodeling with aging and cognitive decline (Wang, 2008; Lu and Hawkins, 2006; Majdi et al., 2007). Further, the cognitive impairment-specific alteration of synaptic protein expression observed here may be associated with the preferential loss of functional high-efficiency synapses (i.e., multiple spine bouton and perforated synapses) implicated in spatial learning and memory formation (Geinisman et al., 1986; Shi et al., 2005; Burke and Barnes, 2006). The dysregulation of plasticity-related proteins exclusively with cognitive decline occurs in addition to age-related alterations in neurotransmission-regulating proteins, and likely exerts a synergistic effect that capitalizes on an already weakened system to create the cognitively impaired phenotype. These results also provide evidence that cognitive decline is not associated with simply a “more aged” phenotype but rather a complex combination of changes specific to cognitive impairment and age-related changes that are not correlated to cognitive status. The identification of cognition-specific hippocampal alterations offers the potential for targeted interventions to prevent or reverse cognitive decline by restoring mechanisms of synaptic plasticity. Future studies examining the effects of manipulating the expression of individual proteins and combinations of proteins in the hippocampus of aged, behaviorally assessed animals will provide more specific mechanistic insight into the functions of these proteins in synaptic plasticity and behavioral performance with cognitive decline.

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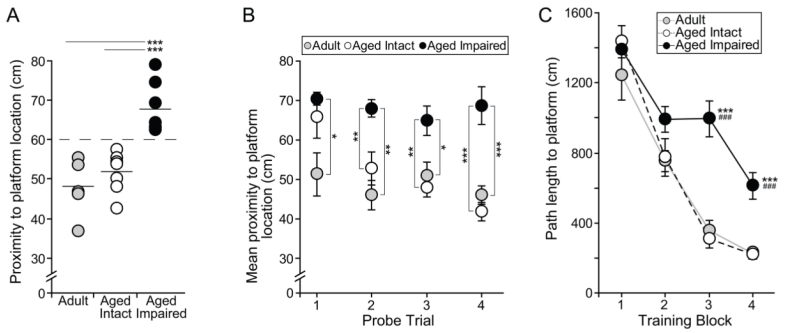

Figure 1.

Behavioral characterization of cognitive decline in Aged rats. Morris water maze testing consisted of acquisition (training) and probe (test) trials. Segregation by cognitive status reflects post hoc classification by probe trial performance. **(A)** Classification of rats by cognitive status was performed using mean performance across multiple probe trials. Aged rats performing within the range of Adult rats were classified as Aged Intact, while those that performed outside this range were classified as Aged Impaired. Based on this stratification, Aged Impaired rats demonstrated significantly poorer cognitive performance than both Adult and Aged Intact rats. Points represent individual animals and horizontal bars indicate group means; *** $p<0.001$. **(B)** Stratification according to cognitive performance was consistent throughout probe testing. Adult rats performed consistently well across probe trials, as indicated by lower proximity values. Aged Intact rats performed similarly to Adult rats, with no significant differences in proximity to the escape platform location between these groups in any probe trial. Aged Impaired rats performed significantly worse than Adults on all four trials and significantly worse than Aged Intact rats on probe trials 2, 3 and 4. Data are presented as mean ± SEM; * $p<0.05$, ** $p<0.01$, *** $p<0.001$. **(C)** Decreasing path length to the escape platform in the acquisition phase of Morris water maze testing indicates the development of a spatially-focused search pattern. Adult (n-5) and Aged Intact (n=8) rats demonstrated marked improvement across the four training blocks. Aged Impaired rats (n-7) performed significantly worse than their Adult and Aged cognitively Intact counterparts on Blocks 3 and 4. Data are presented as mean ± SEM; *** $p<0.001$ vs. Adult, $^{###}$ $p<0.001$ vs. Aged Intact. Statistical analysis: one-way ANOVA with Student Newman Keuls post hoc tests.

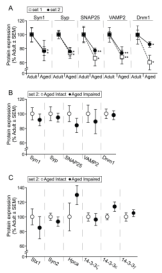

Figure 2.

Differential expression of neurotransmission-regulating proteins with aging but not cognitive decline**. (A)** Expression of a number of synaptic proteins decreases between adulthood and advanced age (set 1, (VanGuilder et al., 2010). These targets were quantitated by immunoblotting in an independent cohort of Adult and Aged rats regardless of cognitive status (set 2). Synaptosomal expression of synapsin 1, synaptophysin, SNAP25, VAMP2, and dynamin 1 was significantly decreased in hippocampal synaptosomes of Aged rats compared to Adults. The magnitudes of these protein alterations are comparable between animal cohorts. **(B)** When Aged rats were segregated by cognitive status into Aged Intact (n=8) and Aged Impaired (n=7) groups, no significant differences in synaptosomal protein expression were detected. **(C)** No cognition-based differences in synaptic expression of additional neurotransmission-regulating proteins differentially expressed with increasing age were observed. Statistical analysis: unpaired, two-tailed t-test: * $p<0.05$, ** $p<0.01$.

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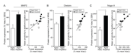

Figure 3.

Synaptic expression of structural remodeling proteins is inversely correlated with cognitive performance in the Morris water maze. **(A)** Microtubule-associated protein 2 (MAP2) expression was significantly elevated in Aged Impaired rats compared to their age-matched cognitively Intact counterparts. MAP2 expression in individual animals demonstrated a significant correlation to cognitive performance assessed by mean proximity to the escape platform location across four probe trials (r= 0.722). **(B)** Expression of drebrin expression was significantly increased in Aged Impaired rats compared to Aged Intact rats and was significantly correlated to mean proximity to the escape platform location (r=0.805). **(C)** Neurite-outgrowth inhibitor A (Nogo-A) expression was significantly elevated in Aged Impaired rats compared to Aged Intact rats. Synaptosomal Nogo-A levels were significantly correlated to mean proximity-to-platform-location measures (r=.690). Statistical analysis: unpaired, two-tailed t-test: * $p<0.05$, ** $p<0.01$; Pearson correlation: $^{\#\#}$ $p<0.01$, $^{\#\#\#}$ $p<0.001$.

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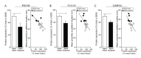

Figure 4.

Synaptic expression of plasticity-related proteins negatively correlates with cognitive performance in the Morris water maze. **(A)** Postsynaptic density protein 95kDa (PSD-95) was significantly decreased in Aged Impaired rats compared to Aged Intact rats. Individual expression levels of PSD-95 demonstrated a significant negative correlation to mean proximity-to-platform measures evaluated in Morris water maze probe trials ($r=-0.632$). **(B)** 14-3-3θ expression was significantly downregulated in Aged Impaired rats compared to Aged Intact rats, and correlated to animals' mean proximity to the escape platform location ($r=-0.539$). **(C)** Calcium/calmodulin-dependent protein kinase 2α (CaMKIIα) was expressed at significantly lower levels in hippocampal synaptosomes from Aged Impaired rats than from Aged Intact rats. CaMK2 expression exhibited a significant negative correlation with mean-proximity-to-platform measures ($r=-0.587$). Statistical analysis: unpaired, two-tailed t-test: * $p<0.05$, ** $p<0.01$; Pearson correlation: $^{\#}$ $p<0.05$.

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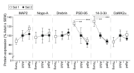

Figure 5.

Expression of plasticity-associated proteins regulated with cognitive decline is not altered with aging. With the exception of PSD-95 and 14-3-3θ, which are expressed at significantly lower levels in Adult and Aged rats than in Young rats (set 1, (VanGuilder et al., 2010), no general age-related alterations in protein expression were detected. Specifically, synaptosomal expression of MAP2, drebrin, Nogo-A, PSD-95, 14-3-3θ and CaMKIIα is unaltered between Adult (n=5–10) and Aged rats (n=15–20). These proteins were expressed at comparable levels in Adult and Aged groups between the two independent animal cohorts. Statistical analysis: one-way ANOVA with Student Newman Keuls posthoc tests: ** $p<0.01$, *** $p<0.001$.

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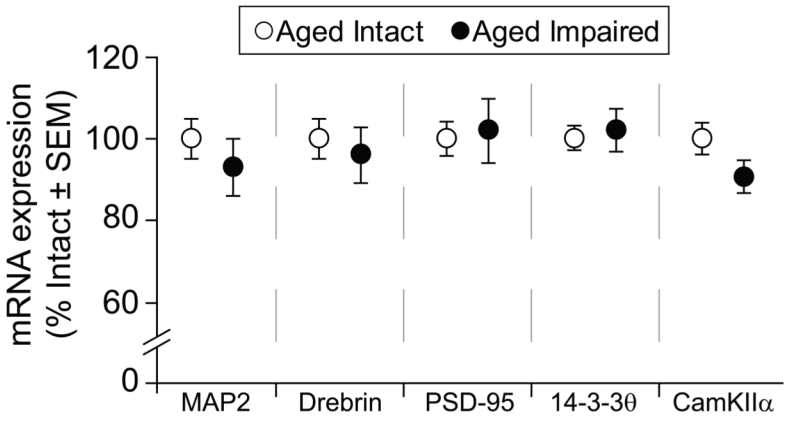

Figure 6.

Cognitive decline-associated proteins are not regulated by local transcript levels. Local mRNA expression of proteins regulated with cognitive status was quantitated by qPCR. No differences in transcript levels were observed. Statistical analysis: one-way ANOVA.

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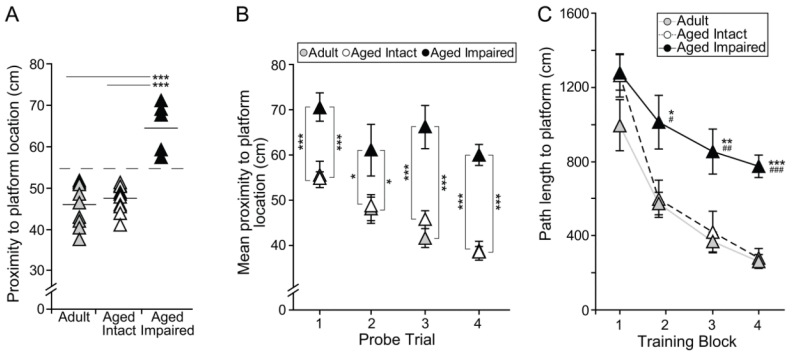

Figure 7.

Stratification of Aged rats by cognitive performance. A second cohort of Adult (n=9) and Aged animals (n=15) was assessed for cognitive status by Morris water maze testing. Groupings reflect post hoc classification by mean probe trial performance. **(A)** Aged Intact rats performed similarly to Adult rats on probe trials, while Aged Impaired rats performed significantly worse. Points represent individual animals and horizontal bars indicate group means; *** $p<0.001$. **(B)** Group is apparent across individual probe trials, with the Aged Impaired group performing significantly worse than the Adult and Aged Intact groups on all probe trials. Data are presented as mean ± SEM; * $p<0.05$, *** $p<0.001$. **(C)** Adult (n=9) and Aged Intact (n=10) rats demonstrated improved performance across training blocks, indicated by decreasing path length to the escape platform. Aged Impaired rats (n=5) performed significantly worse than Adult and age-matched Aged Intact rats on blocks 2–4, and demonstrated a lesser degree of improvement throughout acquisition. Data are presented as mean ± SEM; * $p<0.05$ vs. Adult, $^{\#}$ $p<0.05$ vs. Aged Intact, ** $p<0.01$ vs. Adult, $^{\#\#}$ $p<0.01$ vs. Aged Intact, *** $p<0.001$ vs. Adult, $^{\#\#\#}$ $p<0.001$ vs. Aged Intact. Statistical analysis: one-way ANOVA with Student Newman Keuls post hoc tests.

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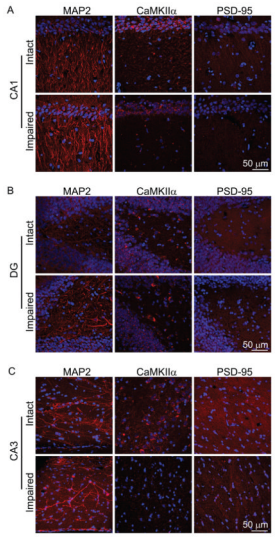

Figure 8.

Effect of cognitive decline on hippocampal MAP2, CaMKIIα, and PSD-95 expression. Differential protein expression in Aged Impaired (n=4) versus Aged Intact (n=4) rats was evident in CA1 (top), DG (middle) and CA3 (bottom) subregions. Increased MAP2 expression was associated with increased dendritic immunoreactivity as well as aberrant dendritic morphology. CaMKIIα immunoreactivity, which was distributed around cell bodies and in synaptic compartments, was decreased uniformly throughout the hippocampus of Aged Impaired animals. PSD-95 staining demonstrated decreased protein expression, as well as an apparent decrease in immunoreactive puncta, with cognitive impairment.

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Table 1

Animal Data

Animal Cohort	Symbol	Cognitive Assessment	Age (months)	Group	n	Sample Type	Biochemical Assessment
Set 1*	■	no	3	Young	10	Hippocampal synaptosomes	Immunoblot analysis of protein targets with aging
			12	Adult	10		
			26	Aged	10		
Set 2	●	yes	12	Adult	5	Hippocampal synaptosomes	Immunoblot/qPCR analysis of targets with cognitive status and aging
			28	Aged Intact	8		
			28	Aged Impaired	7		
Set 3	▲	yes	13	Adult	9	Fixed, frozen sections	Immunohistochemical localization of cognition-related protein targets
			26	Aged Intact	10		
			26	Aged Impaired	5		

* Previously described in (VanGuilder et al., 2010)

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Table 2

Antibody information

Antigen	Supplier	Cat #	host	use	method
14-3-3γ	Cell Signaling Technology[a]	9637	rabbit	primary	IB
14-3-3ε	Cell Signaling Technology[a]	9635	rabbit	primary	IB
14-3-3ζ	Cell Signaling Technology[a]	9639	rabbit	primary	IB
14-3-3θ	Cell Signaling Technology[a]	9638	rabbit	primary	IB
Aldolase C	Santa Cruz Biotechnology[b]	sc12065	goat	primary	IB
CaMKIIα	Cell Signaling Technology[a]	3357	rabbit	primary	IB/IHC
Drebrin	Millipore[c]	ab10140	rabbit	primary	IB
Dynamin 1	Chemicon[d]	mab5402	mouse	primary	IB
Hippocalcin	Abcam[e]	ab24560	rabbit	primary	IB
MAP2	Cell Signaling Technology[a]	4542	rabbit	primary	IB/IHC
Nogo-A	Millipore[c]	ab5888	rabbit	primary	IB
PSD-95	Chemicon[d]	ab18258	rabbit	primary	IB/IHC
SNAP25	BD Transduction Labs[f]	610367	mouse	primary	IB
Synapsin 1	Abcam[e]	ab8-10	rabbit	primary	IB
Synapsin 2	Abcam[e]	ab68850	rabbit	primary	IB
Synaptophysin	Sigma Aldrich[g]	s5768	mouse	primary	IB
Syntaxin 1	Santa Cruz Biotechnology[b]	sc12736	mouse	primary	IB
VAMP2	Synaptic Systems[h]	104211	mouse	primary	IB
HRP × Mouse	GE Healthcare[i]	NXA931	goat	secondary	IB
HRP × Rabbit	GE Healthcare[i]	NA934V	goat	secondary	IB
HRP × Goat	Santa Cruz Biotechnology[b]	Sc2768	rabbit	secondary	IB
DL649 × rabbit	Jackson ImmunoResearch[j]	711495152	donkey	secondary	IHC

[a]Danvers, MA;

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[b]Santa Cruz, CA;
[c]Billerica, MA;
[d]Temecula, CA;
[e]Cambridge, MA;
[f]San Jose, CA;
[g]St. Louis, MO;
[h]Goettingen, Germany;
[i]Piscataway, NJ;
[j]West Grove, PA