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Aged rats with preserved memory dynamically recruit hippocampal inhibition in a local/global cue mismatch environment

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ABSTRACT

Similar to elderly humans, aged outbred Long-Evans rats exhibit individual differences in memory abilities, including a subset of aged rats that maintain memory function on par with young adults. Such individuals provide a basis for investigating mechanisms of resilience to age-related decline. The present study examined hippocampal gene expression in young adults and aged rats with preserved memory function under behavioral task conditions well established for assessing information processing central to the formation of episodic memory. Although behavioral measures and hippocampal gene induction associated with neural activity and synaptic plasticity were similar across age groups, a marker for inhibitory interneuron function in the hippocampal formation was distinctively increased only in aged rats but not in young adults. Because heightened hippocampal neural activity is associated with age-related memory impairment across species, including rats, monkeys, and humans, this finding may represent an adaptive homeostatic adjustment necessary to maintain neural plasticity and memory function in aging.

1. Introduction

The brain is malleable over the course of a lifespan as it develops, incorporates new information, and adapts to an ever-changing environment well into old age. Although memory decline is common, it is not an inevitable consequence of aging. Many older adults maintain high performance throughout life (Duzel et al., 2011; Nyberg et al., 2012). Even in the presence of accumulating pathology that is associated with dementia, an apparent resilience is exhibited in a condition of asymptomatic Alzheimer's disease (AD) when cognitive function is maintained (Arnold et al., 2013; Driscoll and Troncoso, 2011). In that context, the study of elderly individuals with preserved cognitive function may yield important insights into mechanisms that could be broadly applicable to mitigate cognitive decline in aging, even in the presence of significant brain pathology.

Aged outbred rodents, similar to humans and nonhuman primates (Rapp and Amaral, 1991; Stark et al., 2010]), exhibit individual differences in aging for memory that is dependent on medial temporal lobe–hippocampal circuitry (Gallagher et al., 1993; Koh et al., 2014). In the most extensively studied model for individual differences in aged rodents, approximately half of healthy aged Long-Evans rats exhibit preserved cognitive function (Gallagher et al., 1993). These aged unimpaired (AU) rats perform on par with young adult (Y) rats and demonstrate test-retest reliability of intact memory for months after initial characterization (Gallagher and Burwell, 1989; Gallagher et al., 2006; Robitsek et al., 2008). Individual differences defined by performance in spatial memory in this model also extend to tests of hippocampal-dependent nonspatial memory (Gallagher and Burwell, 1989; Robitsek et al., 2008). For example, in assessing contributions of recollection and familiarity to recognition memory as defined by the dual-process model (Yonelinas, 2002), aged rats previously characterized with spatial memory impairment, similar to young adults with hippocampal damage (Fortin et al., 2004), have a selective deficit in recollection with intact item recognition based on familiarity (Fortin et al., 2004; Robitsek et al., 2008). In contrast, aged cohorts with preserved spatial memory exhibit intact recognition based on both recollection and familiarity on par with young adults.

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As reviewed elsewhere (Leal and Yassa, 2015), an emerging understanding of an underlying basis for neurocognitive change within the hippocampal formation has also generalized across mammalian species as a basis for individual differences in aging outcomes. Specific circuits, including the dentate gyrus (DG) and CA3 hippocampal subfields, normally contribute to episodic memory by encoding representations tied to specific events and experiences in a manner that limits interference with similar experiences in the past. During in vivo recordings, unlike Y and AU rats, aged impaired (AI) rats exhibit heightened neural activity in these circuits and neurons fail to rapidly encode distinctive representations of new information (Wilson et al., 2003, 2005). This overactivity has been identified as contributing to age-dependent memory impairment in affected rodents (Haberman et al., 2017; Koh et al., 2010), nonhuman primates (Thome et al., 2016), and humans (Yassa et al., 2011). In the case of activation in the DG/CA3 detected by functional magnetic resonance imaging, greater activation is specifically associated with worse performance due to mnemonic interference in the elderly (Stark et al., 2013; Yassa et al., 2011). This feature, exhibited during aging with memory impairment in the mammalian brain across species, appears to worsen in elderly humans during the prodromal phase of AD (Bakker et al., 2012, 2015).

In the healthy brain, homeostatic mechanisms normally stabilize neuronal excitability and firing properties through a variety of mechanisms, including the recruitment of inhibitory interneurons, to maintain synaptic function in an optimal range for plasticity. Recent evidence indicates that a failure of such homeostatic regulation could contribute to dysfunction in the aged brain as well as in AD (Styr and Slutsky, 2018; Xiao et al., 2017). Several studies have documented age-dependent reductions of interneuron markers (Spiegel et al., 2013; Stanley and Shetty, 2004; Vela et al., 2003) and function (Villanueva-Castillo et al., 2017) in rodents, which, in some instances, correlate with impaired memory performance (Spiegel et al., 2013). In that context, a gain of inhibitory function in AU compared with AI rats is suggested by prior studies in the outbred rat model. Specifically, gene expression profiling of the hippocampal CA3 subfield indicated that induction of inhibitory pathway genes supports cognition in aged rats with intact memory (Haberman et al., 2013). After exposure to a new water maze environment, AU rats have significantly increased expression, relative to aged memory-impaired rats, in a panel of genes related to inhibitory neurotransmission including the primary GABA synthesis enzyme, Gad1. In AU rats, such elevated expression of inhibitory genes occurred alongside the expected induction of synaptic plasticity-related genes. Those findings suggest that inhibitory neurotransmission is recruited to counter abnormal hyperactivity in the hippocampus of aged rats with preserved memory function but not in AI subjects. This recruitment may contribute to normalization of neural excitability and resilience to cognitive decline in aging.

The present study examines this perspective by assessing gene expression measures related to excitation and inhibition in AU rats and young adults in an independent protocol validated for hippocampal recruitment and well studied for hippocampal computational processing. After determination of cognitive status by performance in a standardized Morris water maze protocol, we adapted a cue mismatch task (Knierim, 2002) that engages hippocampal functions critical for memory formation. Here, we report that AU rats show normal induction of gene markers for neural activation and plasticity in response to task contingencies, but unlike young adults, also exhibit elevated induction of markers for the function of inhibitory neurons in hippocampal circuits, consistent with an adaptive mechanism to control excitatory and inhibitory balance.

2. Materials and methods

2.1. Subjects

Aged, male Long-Evans rats were obtained at 8–9 months of age from Charles River Laboratories (Raleigh, NC) and housed in a vivarium at Johns Hopkins University until 24–26 months of age. Young rats obtained from the same source were tested around 4–6 months of age. All rats were individually housed at 25 C and maintained on a 12-hour light/dark cycle. Food and water were provided ad libitum unless indicated otherwise. The rats were examined for health and pathogen-free status throughout the study, as well as necropsies at the time of sacrifice. Rats that showed impaired health or disabilities that could impact behavioral performance (e.g., poor eyesight, clinical evidence of renal impairment, pituitary or other tumors) were excluded from the study. All procedures were approved by the Johns Hopkins University Institutional Animal Care and Use Committee in accordance with the National Institutes of Health directive.

2.2. Behavioral characterization

The aged Long-Evans rat study population shows a larger range of individual differences in memory performance than younger adult control rats, such that some older rats (AU) perform on par with the normative range of younger adult performance (Y), whereas others (AI) perform outside that range (Gallagher et al., 1993; Haberman et al., 2011). To specifically examine hippocampal properties that contribute to intact cognition in aging, we selected only memory unimpaired aged rats for testing alongside young adults in the current experiment. All rats were tested for hippocampal-dependent memory performance in a well-established Morris water maze protocol as described in further detail elsewhere (Gallagher et al., 1993). Training occurred during the light phase, consistent with the standard protocol. Briefly, the water maze consisted of a circular pool surrounded by curtains with large contrasting cues affixed to them. Rats were trained for 8 days (3 trials per day) to locate a camouflaged escape platform that remained at the same location throughout training. Every sixth trial consisted of a probe trial (no escape platform for the first 30 second of the trial) that served to assess the development of a spatially localized search. A memory index was generated from the proximity of the rat to the escape platform during probe trials and was used to distinguish intact performance from memory impairment in the aged rats. The index is the sum of weighted proximity scores obtained during probe trials, with low scores reflecting a more accurate search. A learning index cutoff was used to segregate aged rats into unimpaired (AU, learning index <240) and impaired (AI, learning index >240) such that AU rats fell within the range of young (Y) normative data collected over many years and exhibited by young rats in the present study. Each run cohort included both young and aged rats. Young and AU rats were selected for this experiment from several independent runs with a total of 18 AU and 14 young rats with similar search error and learning indices. A repeated-measures ANOVA confirmed all rats improved with training [RM-ANOVA, trial; $F(3,84) = 115.54$, $p = 0.0001$], but there was no main effect of age [RM-ANOVA, age; $F(1,28) = 1.353$, $p = 0.255$] or interaction [RM-ANOVA, trial x age; $F(3,84) = 1.036$, $p = 0.381$] (Fig. 1B). There was no significant difference between learning index scores of Y and AU [one-way ANOVA (1W-ANOVA); $F(1,28) = 0.104$, $p = 0.75$] (Fig. 1C). Y and AU animals were pseudorandomly assigned to one of two conditions (double rotation or no change, see below) such that memory index scores were similar across the double rotation and no change conditions.

2.3. Double cue rotation task

The double cue rotation protocol was adapted from that used originally by Knierim (2002). Following behavioral characterization, all rats were placed on a food-restricted diet and weighed every day to maintain body weight at 85% of free-feeding weight. When rats reached stable body weight (average 19 days after beginning food deprivation), all Y and AU rats (Y, N = 14; AU, N = 18) were acclimated to run clockwise (CW) on a circular track (76-cm outer diameter, 10-cm width) to collect bacon crumble rewards placed at arbitrary locations on the track. This training occurred during the dark phase beginning at least 1 hour after the lights turned off. While running on the track, the rat was discouraged from turning around and moving counterclockwise (CCW) by blocking its path with a paper folder. By the time of test day, turning behavior occurred infrequently. The track was composed of 4 textured surfaces that served as local cues, each covering a quadrant of the track. The track was placed in a circular, curtained environment (2.7-m diameter) in which 6 distinct peripheral objects were present either on the floor or on the curtain, serving as global cues. Rats were run in 3 cohorts of approximately 12 rats each. For each day, the randomly chosen start point remained consistent for all rats in that cohort. Track acclimation occurred once a day for 10 days with a 1-day intermission after day 5. Each session lasted 20 minutes for days 1–5, after which each session was 20 minutes or 20 laps whichever occurred first. The number of laps completed in each 20-minute training session by each animal was recorded to ensure rats were adequately traversing the track and sampling the environment. The 20-lap limit was introduced to reduce potential differences in activity between young and aged rats, which tended to move more slowly around the track. However, very few rats met this criterion including 3 AU rats and 5 young rats completing 20 laps before 20 minutes by day 10. On the test day (day 11), rats were placed on the track with either the same cue orientation as during the acclimation period (no change [NC]) or with cues rotated (double rotation [DR]) such that local cues were rotated 90 CCW and global cues were rotated 90 CW for a total of 180-degree mismatch. This results in a novel cue configuration where local cues are placed in maximum conflict with the global cues, a manipulation that has been previously shown to impact computational processing and hippocampal encoding (Knierim and Neunuebel, 2016; Lee et al., 2004, 2015; Neunuebel and Knierim, 2014).

2.4. Head scanning analysis

As a behavioral measure to indicate recognition of the cue mismatch in the double rotation condition, we examined head scans for all rats for final acclimation days 9 and 10 and the test day. Scans were manually counted from digital recordings from a camera mounted directly above the track. A head scan was counted when a rat paused in its locomotion around the track and rotated his head side to side such that his nose extended beyond the edge of the track on at least one side. We required that the rat proceed at least 2 steps forward before a second head scan could be recorded. Vertical head movements in the absence of side-to-side movements

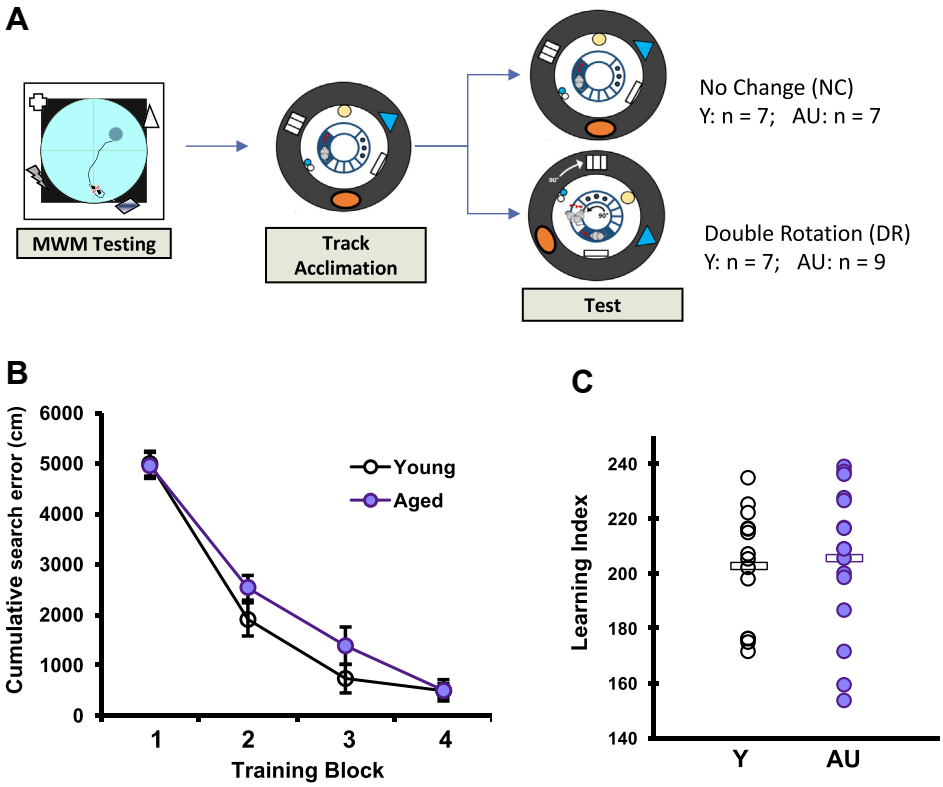

Fig. 1. Performance in water maze behavioral characterization. (A) Schematic of behavioral procedures. Morris water maze (MWM) testing was used to characterize hippocampal-dependent learning ability in young and aged rats. This was followed by 10 days of training on a circular track with both local and global cues in a fixed position. On the 11th day, test day, animals in one condition were exposed to the track with cues located in the same position as during training (no change condition), and in the other condition, global cues were rotated 90 clockwise and local cues rotated 90 counterclockwise to create a 180 mismatch of position relative to training position (double rotation condition). (B) Cumulative search error during water maze training, blocks of 5 trials each. This measure reflects the distance of the rat from the escape platform throughout its search, with higher numbers indicating worse performance. A repeated-measures ANOVA confirmed both young and aged rats improved with training. Data points represent the average for blocks of 5 training trials SEM. (C) Learning index scores were derived from proximity measures during probe trials interpolated throughout training as in Gallagher et al. (1993), with lower scores indicating better performance. The graph illustrates that aged unimpaired rats perform within the range of young rats on this task, and group means (horizontal bars) were not different. See Section 2.2 for statistics.

were not counted as they were difficult to discern given the camera location. Head scans were not counted if the rat was moving in the incorrect (CCW) direction on the track. To control for the variation in the amount of time rats were on the track, scanning rate was computed by dividing the total number of head scans for the session by the amount of time spent on the track during that session.

2.5. In situ hybridization and analysis

Rats were perfused 2 hours from the beginning of each rat's test day session with phosphate buffered saline followed by 4% paraformaldehyde. Brains were stored overnight in 4% paraformaldehyde and then cryoprotected in 20% sucrose. Brains were cut in the coronal plane at 40 μm using a freezing microtome and stored in a 1 in 24 series in 4% paraformaldehyde until processed for mRNA by in situ hybridization. Quantitative in situ hybridization procedures and probe generation were performed as in the study by Haberman et al. (2011). Briefly, brain sections matched for number and location were hybridized overnight at 60 C in buffer containing a ^{35}S-UTP-labeled riboprobe generated using the MAXIscript kit (Ambion). In situ probe sequences were either PCR amplified from whole hippocampal cDNA with primers incorporating T7 and SP6 RNA polymerase binding sites or PCR-derived amplicons were cloned in pGEM plasmids and digested with appropriate restriction enzymes. Probe sequences were as follows: Gad1, nts 268 to 574 of GenBank sequence NM_017007.1; Zif268, nts 770-1143 from GenBank seq NM_012551.2; Tubg1, nts1252-1601 of GenBank seq NM_145778.2; and cFos, nts 670 to 1043 from GenBank seq NM_022197.2. Nlgn1 and Camk2a probes were described previously (Haberman et al., 2008). All probes were verified for specificity by BLAST search of the fragment sequence. The specificity of Zif268 and Gad1 probes were further confirmed by competition assay with gene family members, Egr2 and Gad2, respectively. No cross competition was detected with these sequences. Mounted, dried sections were exposed in a phosphorimager cassette. Brain regions of interest were outlined by hand and matched for level along the anterior-posterior axis and quantified, blind to experimental conditions, using ImageQuant (GE Healthcare, PA). Radioactive standards exposed at the same time as the brain sections ensured that section intensity was within the linear range and all intensity values were normalized to these standards. Typically, 4 intensity measurements per animal for each area of interest were averaged to obtain a single score for each rat. Expression values for each rat of Zif268, Camk2a, Nlgn1, and Gad1 were normalized to Tubg1 levels, which shows no difference between Y and aged rats in either basal or activated conditions in previous work (Haberman et al., 2011, 2013) or in the present study. The areas analyzed included whole dorsal hippocampus as well as CA3, CA1, and DG hippocampal subfields (extending from approximately 2.8 mm to 4.16 mm relative to bregma).

2.6. Gad67 immunohistochemistry

Cryoprotected tissue sections from the same subjects used for in situ hybridization were matched for level and stained for Gad67 as in the study by Spiegel et al. (2013) in a single run. Digital 5 images were inverted, and the CA3 subfield and dentate granule layer (both dorsal and ventral blades) were manually outlined under blinded conditions using ImageJ to obtain overall intensity for the area of interest. As per in situ hybridization, 4 regions of interest (ROIs) per animal covering the CA3 region or dentate granule cell layer were averaged to obtain a single score for each rat. Slide background levels were taken for each ROI and subtracted from mean intensity values. Data were analyzed by 1W-ANOVA. The same CA3 ROIs were analyzed for the number of Gad67-positive cells with exhaustive counting using Stereo Investigator (MBF Bioscience, Williston, VT, USA). Using the optical fractionator process within Stereo Investigator, 100% of each ROI was counted using a 40X objective. The number of positive cells per ROI was averaged across 4 ROIs per subject. Tissue sections for 2 AU rats were unavailable for cell counting, and therefore for this experiment, group numbers were AU DR: n = 8, AU NC: n = 6, Y DR: n = 7, and Y NC: n = 7. A 2-way ANOVA (2W-ANOVA) was used to assess main effects, followed by 1W-ANOVAs between conditions.

2.7. Experimental design and statistical analysis

As the experiment was designed to assess hippocampal gene activation of AU rats under conditions intended to engage hippocampal computational processes, young rats were included as a comparison group to determine normative gene induction under these behavioral conditions. The NC behavioral condition was included to control for hippocampal mRNA modulation due to the physical performance of the task and exposure to the environment. Therefore, all gene measures were normalized to this condition within each age group. Figures illustrating gene expression are presented as percentage of NC average. To obtain these data, intensity values were normalized to the average intensity in the NC condition observed in the ROI within each age group, and values are expressed as the mean SEM. For the behavioral measures, data are presented as within-subject measures (change from baseline performance, see below).

For the behavioral analysis, we recorded the number of laps run during the acclimation period and test days (days 1–11), and head scans across days 9, 10, and test day. Two rats from the aged group in the NC condition failed to demonstrate adequate performance during acclimation, performing 2 standard deviations below the mean for both laps and scans when compared across all animals, as well as within the NC condition alone. Therefore, data for those animals were excluded from all analyses. This resulted in the following group sizes: Y NC = 7, Y DR = 7, AU NC = 7, and AU DR = 9.

Test day behavioral measures were expressed as a percent of baseline, defined as each subject's average performance on the last 2 acclimation days before test day. There were no differences in baseline measures of lap running or head scanning between age groups or behavioral conditions (data not shown). In addition, test day changes (relative to baseline) in the DR condition did not correlate with learning index for all subjects (Pearson, n = 30; Laps: r =− 0.1465, p = 0.44; Scans: r =− 0.0662, p = 0.73; Scans/min: 0.0195, p = 0.92) or independently for age group or behavioral measure. For all comparisons of behavioral measures between groups on test day, 2W-ANOVAs were run with age (Y and AU) and test condition (NC or DR) as between-subject factors. Follow-up comparisons were conducted by either 1W-ANOVA or paired t-tests, as appropriate. Levene's test was used to determine if equal variances were assumed in t-test calculations.

Experiments for Zif268, Camk2a, and Nlgn1 in situ hybridization were run in 2 batches, whereas Gad1 and Tubg1 were run in a single batch. Therefore, all intensity values were normalized to standards for each batch and then converted to z-scores within each batch before running statistical analyses for all genes. Expression levels of each gene were analyzed independently for each ROI and each age group. For each gene assessed, the z-score for each animal in each ROI was used as the dependent variable in a 2W-ANOVA with age and test condition (NC, DR) as between-subject factors. Pearson correlations were used to assess the relationship between variables with Fisher's Z-transformation to test for differences between correlations. Statistical tests used to analyze each data set are noted in the corresponding Results section, and statistical comparisons with p values of <0.05 are considered significant. All statistical analysis was conducted using SPSS PASW Statistics (version 24.0, IBM, Chicago, IL, USA).

3. Results

3.1. Cognitively normal aged rats perform on par with young in the hippocampal-dependent cue mismatch task

Memory index scores, a robust measure of water maze performance that reflects hippocampal integrity, did not differ between the young (n = 14) and AU rats (n = 16) used in this study (Fig. 1 and see details in Section 2.2). After the initial water maze characterization, we adapted a cue mismatch task (Knierim, 2002) that engages hippocampal functions critical to memory formation (schematically shown in Fig. 1A, and details in Section 2.3). All Y and AU rats were acclimated to run on a circular track for 10 days, followed by a test day in which rats experienced either a cue rearrangement (double rotation condition, DR), such that local cues (on the track) and distal cues (surrounding the track) were rotated in opposite directions (90 each for a 180 mismatch), or the familiar cue arrangement (no change condition, NC). This paradigm was designed and previously used to probe hippocampal computational functions (Knierim and Neunuebel, 2016; Knierim and Rao, 2003; Neunuebel and Knierim, 2014). Laps run and head scanning were monitored during the test day session relative to baseline at the end of track acclimation. Head scanning behavior in this task has previously provided a measure of investigatory behavior associated with hippocampal spatial encoding (Monaco et al., 2014).

As shown in Fig. 2 and Supplementary Fig. 1, behavioral measures of performance, including number of laps run, head scans, and scanning rate, differed by test condition for both age groups. During the test session, both young and aged DR animals responded to the cue mismatch with an increase in the number of laps run relative to NC [2W-ANOVA, condition: $F(1,26) = 6.601$, $p = 0.016$; age: $F(1,26) = 0.007$, $p = 0.934$; condition x age: $F(1,26) = 0.008$, $p = 0.929$] (Fig. 2A) and relative to their baseline running behavior (Paired *t*-test: Y DR $p = 0.053$; AU DR $p = 0.014$). In contrast, Y and AU rats in the NC condition ran similar numbers of laps during baseline and test (Paired *t*-test: Y NC $p = 0.796$; AU NC $p = 0.832$) (Fig. 2B). Cognitive engagement, indicated by a head scanning behavioral response, also significantly increased in both young and aged rats in the DR condition relative to NC [2W-ANOVA, condition: $F(1,26) = 16.761$, $p = 0.0001$; age: $F(1,26) = 0.017$, $p = 0.898$; condition age: $F(1,26) = 0.687$, $p = 0.415$] (Fig. 2C). Again, DR rats increased their scanning relative to baseline, whereas animals in the NC condition did not (Paired *t*-test: Y NC $p = 0.561$; Y DR $p = 0.009$; AU NC $p = 0.746$; AU DR $p = 0.0001$) (Fig. 2D). Similar results were found for scanning rate (Fig. 2E) [2W-ANOVA, condition: $F(1,26) = 12.825$, $p = 0.001$; age: $F(1,26) = 0.015$, $p = 0.904$; condition age: $F(1,26) = 0.286$, $p = 0.597$; Paired *t*-test: Y NC $p = 0.482$, Y DR 0.028; AU NC $p = 0.905$, AU DR $p = 0.001$) (Fig. 2F). Altogether, these data show that AU rats responded similarly to young rats during training and test, consistent with other studies demonstrating intact information processing and memory function in aged rats behaviorally characterized as unimpaired in this study population (Gallagher and Burwell, 1989; Haberman et al., 2013; Pereira et al., 2015; Robitsek et al., 2008). The equivalent behavioral response in this cue mismatch paradigm provides a basis to compare hippocampal gene expression signatures between Y and AU.

3.2. Young and AU rats show similar induction of neural activity and synaptic plasticity markers with cue rotation

Gene expression profiles induced in behavioral paradigms can reflect neurobiological processes representing neural activation and synaptic plasticity in which mRNA induction is required for subsequent maintenance and behavioral expression of memory. To investigate the relationship between the cue mismatch and hippocampal network activation, we measured hippocampal Zif268 mRNA, a gene induced by neural activity (Cole et al., 1989), by quantitative in situ hybridization. Zif268 expression (Fig. 3A–F, Supplemental Fig. 2) was elevated in whole hippocampus in animals that experienced the DR condition relative to animals in the NC condition (Fig. 3A) [2W-ANOVA, condition: $F(1,26) = 24.01$, $p = 0.0001$; age: $F(1,26) = 0.128$, $p = 0.724$; condition x age: $F(1,26) = 0.668$, $p = 0.421$]. This difference was confirmed by analysis of cFos, a commonly used indicator of neural activity (Supplemental Fig. 3) (Joo et al., 2016). Both Y-DR and AU-DR rats showed increased Zif268 expression in the principle cell layers of individual hippocampal subfields relative to NC (Fig. 3B–D) [2W-ANOVA, condition: CA3 $F(1,26) = 27.99$, $p = 0.0001$; CA1 $F(1,26) = 15$, $p = 0.001$; DG $F(1,26) = 12.031$, $p = 0.002$, no main effect for age or condition age interaction for any subfield]. These differences were significant in all regions except Y in CA1, which represented a trend [1W-ANOVA, Y DG: $F(1,12) = 6.84$, $p = 0.023$; AU DG: $F(1,14) = 5.334$, $p = 0.037$; Y CA3: $F(1,12) = 9.194$, $p = 0.01$; AU CA3: $F(1,14) = 21.156$, $p = 0.0001$; Y CA1: $F(1,12) = 4.012$, $p = 0.068$; AU CA1: $F(1,14) = 4.012$, $p = 0.003$]. Furthermore, hippocampal Zif268 expression correlated with the increase in scans (percent baseline) on test day for all subjects (Fig. 3E) ($r = 0.423$, $p = 0.020$) as well as within each age group (Y: $r = 0.536$, $p = 0.048$; AU: $r = 0.559$, $p = 0.024$).

We also examined the effect of cue mismatch on downstream synaptically localized, plasticity-related transcripts. In young adults, both Camk2a and Nlgn1 are increased in the CA3 subfield in response to spatial learning (Haberman et al., 2008). Consistent with this previous work, in situ hybridization assessment of CA3 showed increased expression of both CamK2a [Fig. 3G and H: 2W-ANOVA, condition: $F(1,26) = 17.203$, $p = 0.0001$; age: $F(1,26) = 0.05$, $p = 0.825$; condition age: $F(1,26) = 0.337$, $p = 0.566$] and Nlgn1 [Fig. 3I and J: 2W-ANOVA, condition: $F(1,26) = 18.913$, $p = 0.0001$; age: $F(1,26) = 0.078$ $p = 0.782$; condition age: $F(1,26) = 0.013$, $p = 0.908$]. These data show that, in both age groups, the double cue rotation condition engages not only markers of neural activity but also recruits mechanisms specifically involved in synaptic plasticity and maintenance in the CA3 subfield, both of which are critical to long-term memory (Haberman et al., 2008).

3.3. AU animals activate inhibitory gene, Gad1, alongside neural activity markers

Although our results to this point support comparable behavioral responses and gene induction between young and AU rats, our previous work has demonstrated that AU animals exhibit gene expression signatures of elevated inhibitory control relative to impaired rats in the hippocampus during learning tasks (Haberman et al., 2013). To examine inhibitory activation in this protocol, we assessed Gad1 mRNA expression in the hippocampus in response to the cue mismatch (Fig. 4) and found that AU-DR rats displayed a striking increase over AU-NC, which was not observed in Y rats. In all 3 hippocampal subfields, there was an interaction between age and behavior, but no main effect of behavior or age in any subfield (Fig. 4A–C) [2W-ANOVA, age behavior condition, CA3: $F(1,26) = 12.674$, $p = 0.001$; DG: $F(1,26) = 5.244$, $p = 0.03$; $p = 0.001$; CA1: $F(1,26) = 12.157$, $p = 0.002$]. Follow-up analyses showed that double cue rotation had no significant effect on Gad1 expression in young DR rats relative to NC in CA3 and DG and a small but significant decrease in CA1 [CA3: $F(1,12) = 2.379$, $p = 0.149$; DG: $F(1,12) = 0.234$, $p = 0.637$; CA1: $F(1,12) = 4.358$, $p = 0.03$]. In contrast, AU-DR rats showed a significant increase in Gad1 mRNA relative to NC across all 3 regions [CA3: $F(1,15) = 14.84$, $p = 0.002$; DG: $F(1,15) = 10.67$, $p = 0.007$; CA1: $F(1,15) = 6.14$, $p = 0.027$]. In addition, CA3 Gad1 mRNA showed a strong positive correlation with test day increases in scanning behavior (Fig. 4D) in AU rats (Pearson r =

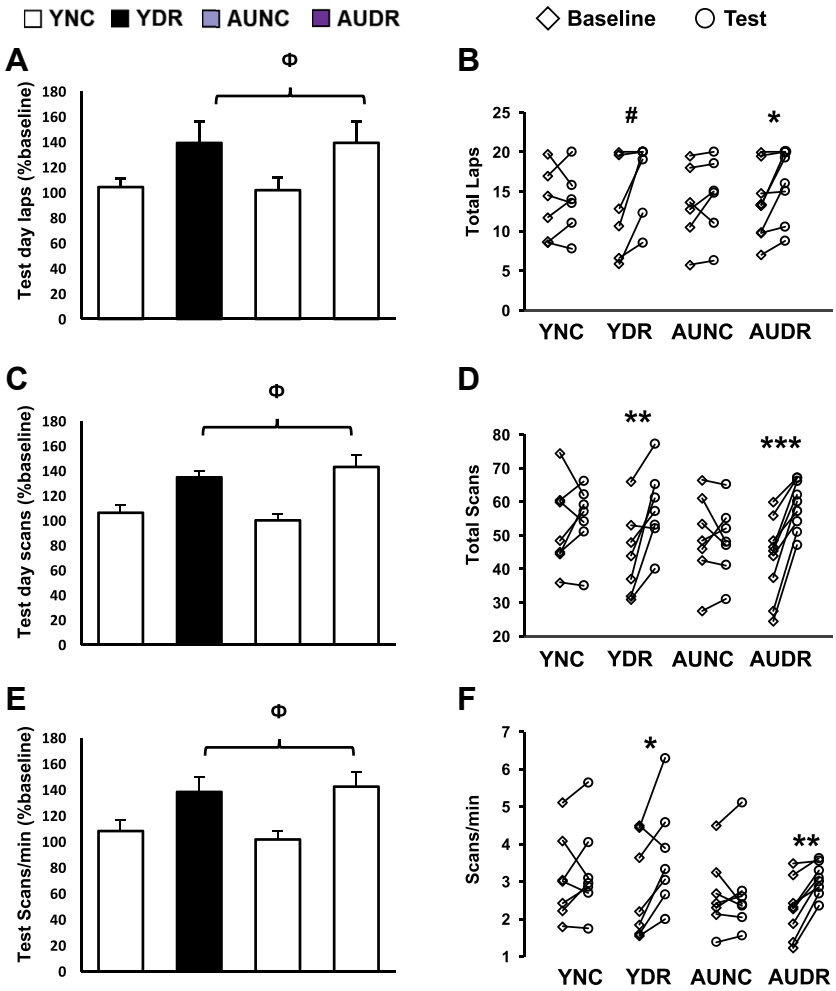

Fig. 2. Double cue rotation task performance was similar in young (Y) and aged unimpaired (AU) rats. On test day, both Y and AU animals in the double rotation (DR) condition responded to the cue manipulation with enhanced exploratory behavior relative to animals in the NC condition. (A and B) Number of laps run. (C and D) Number of head scans. (E and F) Scanning rate (total scans per rat/duration of session (min). (A, C, and E) show group averages as a percentage of baseline SEM; Φ, $p < 0.05$, 2W-ANOVA main effect of behavioral condition. (B, D, and F) illustrate baseline performance (diamonds) and test day performance (circles) for each animal in each group. $^{\#}p < 0.06$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, paired *t*-test of baseline versus test day.

0.830; $p = 0.0001$) but not Y (Pearson r =– 0.269; $p = 0.35$). These correlations were significantly different from each other (Fisher's Z-transformation: Z = 3.57, $p = 0.0004$). Similarly, Gad1 expression correlated with Zif268 expression (Fig. 4E) in AU (Pearson r = 0.648, $p = 0.007$) but not Y (Pearson r =– 0.182, $p = 0.534$) and were likewise significantly different from each other (Fisher's Z-transformation: Z = 2.33, $p = 0.020$). Similar patterns were found for the CA1 and DG subfields, although not all AU correlations were significant (Supplemental Fig. 4). These data suggest that Gad1 mRNA is dynamically elevated in response to hippocampal engagement in AU but not Y rats. The CA3 correlation with a behavioral measure of cognitive engagement suggests Gad1 may be a key component of hippocampal spatial processing in AU rats.

To determine whether there was a corresponding increase in Gad67 protein, the product of the Gad1 gene, we performed immunohistochemical analysis of Gad67 on tissue sections from the same rats (Fig. 4F–I). Although the time point for sacrifice was selected to optimize mRNA intensity, analysis of Gad67 showed trends similar to mRNA effects in CA3 and DG subfields (2W-ANOVA for CA3, age: F(1,26) = 4.195, $p = 0.051$; behavior: F(1, 26) = 2.664, $p = 0.115$; age behavior: F(1,26) = 2.403, $p = 0.133$; for DG, age: F(1,26) = 3.181, $p = 0.086$; behavior: F(1.26) = 1.752, $p = 0.197$; age behavior: F(1,26) = 3.181, $p = 0.086$). Increased immunoreactivity was found in AU-DR rats relative to AU-NC in CA3 (1W-ANOVA: F(1,14) = 7.121, $p = 0.018$) and DG subfields (1W-ANOVA: F(1,14) = 7.07, $p = 0.019$). Consistent with the mRNA analysis, Y rats did not show any differences between DR and NC conditions in immunohistochemical measures (CA3: F(1,12) = 0.002, $p = 0.961$; DG: F(1,12) = 0.076, $p = 0.788$). The detected increase in Gad67 intensity appears to be due to an increase in the number of detectable neurons in AU-DR animals relative to AU NC, as indicated by a near significant trend toward an age behavior interaction (Fig. 4G) [2W-ANOVA, behavior: F(1,24) = 0.395, $p = 0.536$; age: F(1,24) = 2.729, $p = 0.112$; age behavior F(1,24) = 3.958, $p = 0.058$]. Post hoc tests showed a significant difference between the number of cells in AU DR relative to AU NC [F(1,12) = 10.557. $p = 0.007$], but no difference between conditions in Y animals [F(1,12) = 0.042, $p = 0.842$]. The consistency in trends between mRNA and protein measures in the AU rats support a functional consequence of the mRNA induction.

4. Discussion

The present study builds on previous findings demonstrating recruitment of inhibition in aged rats with preserved hippocampal-

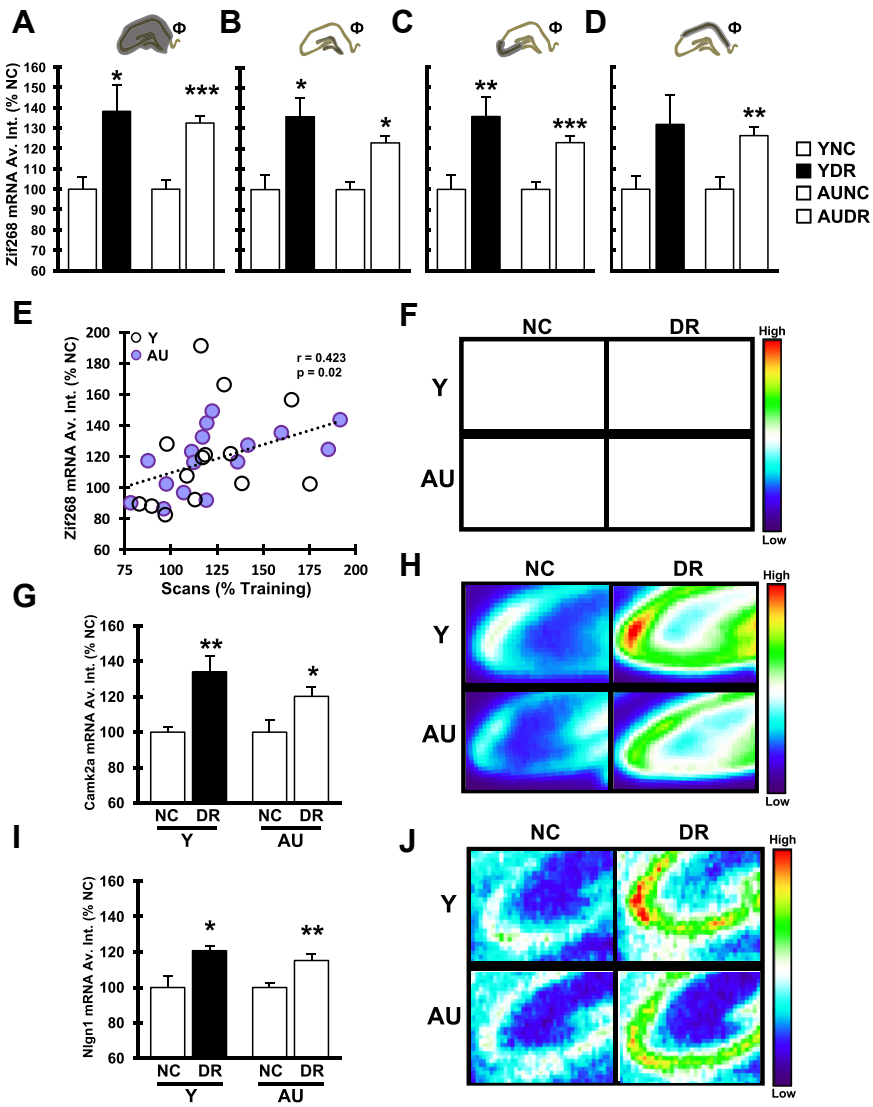

Fig. 3. Elevation of activity- and plasticity-related gene expression in the hippocampus following double cue rotation. Quantification of Zif268 in situ hybridization signal intensity in the dorsal hippocampus showed increased expression of Zif268 in young (Y) and aged DR animals relative to NC controls when assessed in the (A) whole hippocampus, (B) dentate gyrus, (C) CA3, and (D) CA1 hippocampal subfields. Intensity measures are normalized to the average of NC condition for each age and region. (E) Individual values for Zif268 expression for each animal (N = 30, all NC and DR rats) in the whole hippocampus are significantly correlated to the increase in scans on test day. Test day scans are shown as percent baseline, and Zif268 data are normalized to NC condition for each age group. (F) Representative heatmap images of Zif268 in situ hybridization as detected by phosphorimager with corresponding color map. (G) Expression intensity of Camk2a in situ hybridization was measured in the CA3 subfield and demonstrates higher expression in DR subjects relative to NC for both Y and AU groups. (H) Representative heatmap of Camk2a expression. (I) A similar increase in DR relative to NC was found for Nlgn1 expression in CA3. (J) Representative heatmap of Nlgn1 expression. All bar graphs show average SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ for DR versus NC, Φ 2W-ANOVA main effect of behavioral condition. Abbreviations: AU, aged unimpaired; DR, double rotation; NC, no change; Y, young.

dependent cognitive function (Haberman et al., 2013). Here, we specifically examined gene expression measures related to inhibition, as well as neural activity and synaptic plasticity, in AU rats in a cue mismatch paradigm modified to optimize gene expression of the targeted mRNAs. The cue mismatch paradigm was originally developed to investigate computational functions of the hippocampus through recording of single unit responses under varying degrees of cue rotation (Knierim, 2002). The cue mismatch generates a conflict between prior representations of a familiarized environment and the current environment. Prior studies in young animals have reported responses to cue mismatch including changes in neuronal firing rates and place field remapping such that new representations are encoded to minimize interference with previously encoded representations (Knierim and Neunuebel, 2016; Lee et al., 2004, 2015; Neunuebel and Knierim, 2014). When neural properties have been assessed in relation to aging and cognitive impairment in response to environmental to cue manipulation, AI animals display distinct alterations in electrophysiological responses, whereas AU animals respond similarly to young animals, suggesting intact encoding in AU animals (Tanila et al., 1997; Wilson et al., 2003, 2005). Thus, this study focuses on AU animals in comparison with young to examine mechanisms of intact hippocampal encoding in aging.

As expected, AU rats performed similarly to young adult rats on the 3 behavioral measures that differed according to the test condition (DR vs NC): laps, head scans, and scanning rate. The similar behavioral response of young adult and AU rats is consistent with many other studies demonstrating intact information processing and memory function in aged rats characterized as unimpaired by the standardized water maze protocol that has long been used in this study population. We have found that the water maze learning index measure robustly correlates with behavioral performance in other hippocampal-dependent tasks (Haberman et al., 2013;

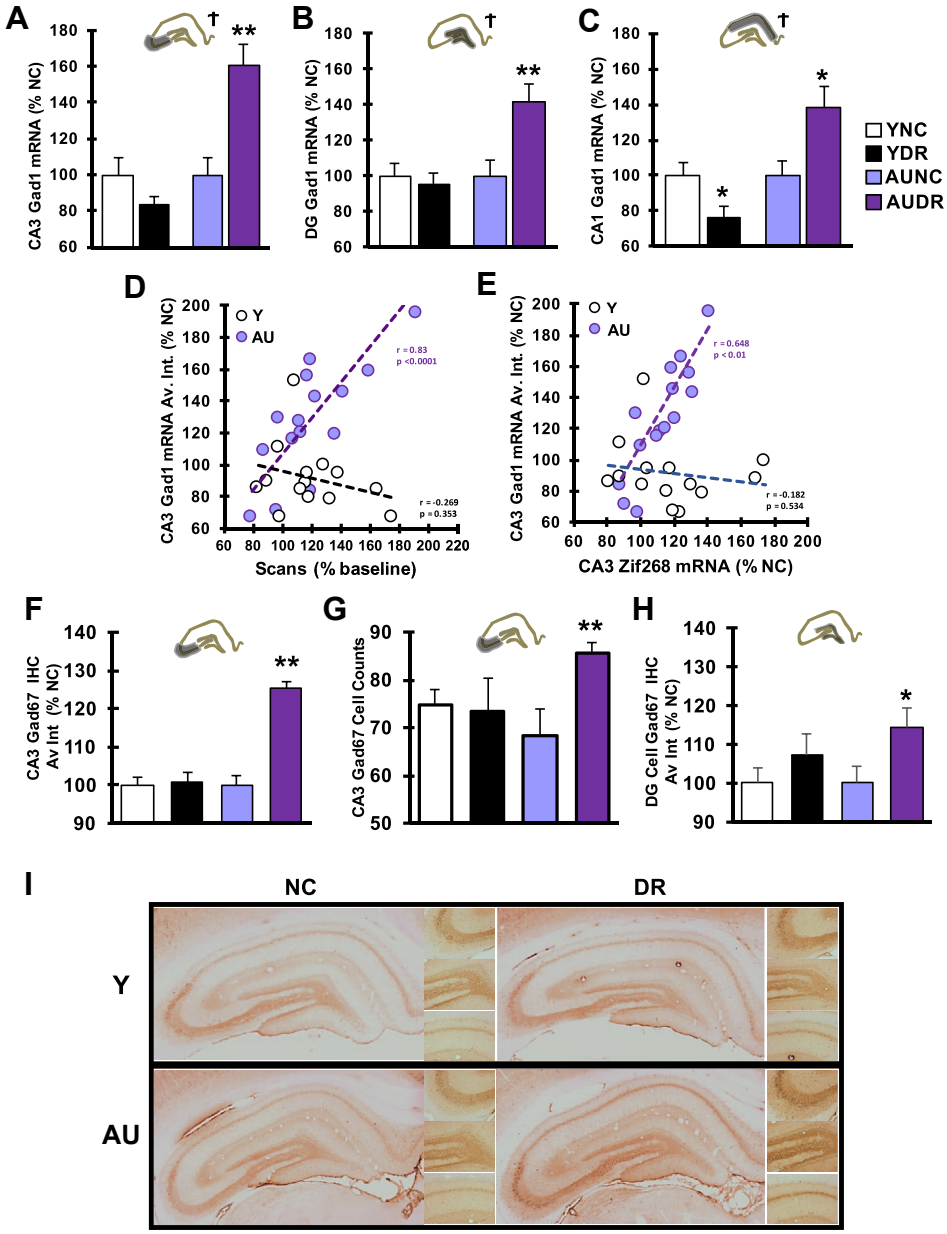

Fig. 4. AU-DR rats upregulate Gad1 mRNA. (A) Quantification of Gad1 in situ hybridization signal intensity in CA3 demonstrates higher expression in AU-DR animals relative to all other groups. Expression intensity of Gad1 mRNA measured in the (B) dentate gyrus and that of in the (C) CA1 subfield also show significantly higher expression in AU-DR subjects relative to all other groups. In a 2W-ANOVA, there was no main effect of behavior condition or age for any subfield, but there was an interaction between age and behavior in all 3 subfields (†). Significance of subsequent, one-way ANOVAs is indicated by asterisks. (D) Individual values for Gad1 mRNA expression in CA3 for each animal show a significant correlation with percent of baseline scans on test day for AU rats but not Y rats. Linear trend lines are based on values for each age group. Correlations for Y and AU were significantly different from each other. (E) Individual values for Gad1 mRNA expression in CA3 for each animal show a significant correlation with CA3 Zif268 mRNA expression again only in AU rats. Linear trend lines are based on values for each age group, and the correlations are significantly different. (F) IHC analysis for Gad67 protein in CA3 showed a near significant main effect of age. Subsequently, one-way ANOVAs within each age group showed that only AU animals displayed a significant increase in Gad67 in the DR condition. (G) Counts of Gad67-positive cells in whole CA3 showed enhanced numbers of Gad67 expressing cells in the AU-DR animals. (H) IHC analysis for Gad67 protein in the DG cell layer showed similar results as CA3. (I) Representative images of Gad67 immunolabeled hippocampal sections for all groups. All bar graphs show average ± SEM; †, 2W-ANOVA interaction between behavioral condition and age; $^{*}p < 0.05$, $^{**}p < 0.01$ for DR versus NC post hoc tests. Abbreviations: AU, aged unimpaired; DR, double rotation; IHC, immunohistochemical; NC, no change; Y, young.

Pereira et al., 2015; Robitsek et al., 2008) and has high test/retest reliability over time (Gallagher and Burwell, 1989; Gallagher et al., 1993). Thus, it is not surprising that the behavior of AU rats was similar to young rats in the present study, with laps, head scans, and scan rate induced across both age groups in response to the double cue rotation manipulation. The increase in head scanning observed in the present study is particularly notable as recent data suggest head scanning at a particular position on the track is associated with the development of a place field at that location on the next traverse of the track in young rats (Monaco et al., 2014). Thus, head scanning represents an opportunity for the rat to encode features of the current environment in addition to serving as a behavioral indicator of information processing with reference to previously experienced spatial and environmental cues.

By comparing gene expression levels from the cue rotation group (DR condition) to a control group with a familiar cue

orientation (NC condition), we found that induction of hippocampal gene expression was clearly tied to a change in the configuration of cues. The induction of Zif268 in both young and AU rats in response to the cue mismatch indicates a similar hippocampal activation in that condition across age groups. Such induction is consistent with multicellular recording evidence that both young and AU rats exhibit rapid encoding of new information when rats, familiarized in one environment, are exposed to a novel environment (Wilson et al., 2003). In addition to providing a marker for neural activity, Zif268 has a demonstrated role in induction of synaptic plasticity and long-term memory (Duclot and Kabbaj, 2017; Jones et al., 2001) and prompted a direct examination of genes whose products mediate synaptic plasticity.

In the context of hippocampal contributions to episodic memory, prior studies using the outbred rodent model have focused on basal and activated gene expression profiles in the CA3 subfield (Haberman et al., 2008, 2011, 2017). In young rats, a cluster of LTP/synaptic plasticity-related genes, including both Camk2a and Nlgn1, is induced in CA3 in the setting of new spatial learning that occurs in a time frame similar to that used in the present study (Haberman et al., 2008). Induction of these genes contributes to the encoding and consolidation of memory as assessed by siRNA knockdown during hidden platform water maze training; siRNA knockdown of Nlgn1 in the CA3 subfield impaired performance on a probe test at 48 hours, whereas rats injected with a control siRNA showed significant spatial bias for the trained platform location. These data suggest that behaviorally induced gene expression is required for the long-term memory of an event. The present study examined Nlgn1 along with CamK2a and found that both mRNAs were induced with the cue mismatch procedure in both young and AU rats. The consistency of gene induction across Y and AU rats supports not only intact neural activation across age groups but also similar mechanistic engagement and induction of relevant synaptic plasticity molecules tied to memory.

A notable finding in the present study was that Y and AU rats differed in the induction of Gad1 in response to the change in the environment in the DR test condition. The selective increase in inhibitory gene expression in AU rats is consistent with previous findings of gene induction in AU rats relative to AI rats during a spatial memory task (Haberman et al., 2013); this study found not only an overall increased gene induction capacity in AU over AI subjects but greater induction specifically for a panel of genes associated with inhibitory function, including Gad1. Recent electrophysiological data have also provided evidence for augmented hippocampal inhibitory function in AU rats compared to both AI and Y rats (Tran et al., 2018), consistent with the earlier gene expression results. These studies, combined with the current findings, suggest inhibitory recruitment is unique to AU. Based on extensive work in AI rats, including findings from electrophysiological recording studies, we would not expect a similar response from these subjects, as they consistently show blunted expression of inhibitory markers relative to AU (Haberman et al., 2011, 2013; Spiegel et al., 2013) and altered neural responses to cue manipulation (Tanila et al., 1997; Wilson et al., 2003). In the present study, recruitment of inhibitory gene expression in AU rats occurs alongside gene induction typical of young rats that together may contribute to preserved memory performance in aging. Indeed, other recent research has directed attention to mechanisms for control of network excitability in human aging and early stages of AD in which recruitment of inhibition appears to represent an age-dependent resilience factor (Xiao et al., 2017).

The inhibitory gene induction by AU subjects observed in the present study is of particular interest based on hippocampal localization of elevated neural activity associated with age-related memory impairment identified across animal models (Simkin et al., 2015; Thome et al., 2016; Wilson et al., 2005) and detected in elderly humans by task-activated functional magnetic resonance imaging affecting the CA3/DG regions (Yassa et al., 2010, 2011). Homeostatic regulation of neural activity in CA3 and DG regions is critical for limiting interference between new representations and similar past representations by a process referred to as pattern separation. In aged individuals, increased hippocampal neuronal activity is tied to memory impairment in both aged rodents and humans by shifting computational processes, such that the distinction between old and new representations is reduced (Stark et al., 2013; Wilson et al., 2005). Enhancement of inhibition to mitigate such heightened activity has a documented cognitive benefit in both preclinical animal and clinical human studies (Bakker et al., 2012; Koh et al., 2010; Sanchez et al., 2012) and improves performance of aged mice in a task designed to test hippocampal pattern separation (Guo et al., 2018). Thus, homeostatic regulation of E/I balance by greater engagement of inhibitory function, particularly under conditions that strongly engage hippocampal activation as demonstrated here, could be adaptive in the aged brain as distinct from young.

Previous research on therapeutics provides some evidence for age-dependent effects of agents that modulate inhibitory function. The use of $GABA_A$-α5-positive allosteric modulators to boost inhibitory function has shown preclinical efficacy in the context of age-related memory impairment (Koh et al., 2013). Based on the high expression of $GABA_A$ α5 receptors in the hippocampus, a novel class of $GABA_A$-α5-negative allosteric modulators (referred to as inverse agonists), which would heighten excitability, was previously reported to provide modest behavioral improvement in young adult rodents (Atack et al., 2006; Ballard et al., 2009; Chambers et al., 2003; Collinson et al., 2006; Dawson et al., 2006); these preclinical studies, however, failed in translational studies of age-related cognitive impairment (Atack, 2010). In the study by Koh et al. (2013), the use of both negative and positive modulators of $GABA_A$ α5 in aged memory-impaired and young adult rats confirmed that negative modulation of $GABA_A$ α5 enhanced cognition in young animals, and conversely, positive modulation benefited cognition in the AI rats. Based on the existing evidence for deficits in hippocampal encoding associated with heightened neural activity in AI rats, and the current findings comparing Y and AU rats, further studies could test the hypothesis that recruitment of inhibitory function plays a distinctive role in the aging brain by comparing the effects of such agents in Y and AU rats. Indeed, the testing protocol used in the present study could be especially well suited for such an analysis, allowing for behavioral and electrophysiological assessment of episodic encoding by hippocampal neurons (Monaco et al., 2014). Based on the study by Koh et al. (2013), it would be predicted that positive allosteric modulation of $GABA_A$ α5 would improve the encoding properties of neurons in age-related memory impairment (AI) rats, consistent with an inability to engage the additional inhibitory recruitment as demonstrated by AU animals. In contrast, given the beneficial effects of negative allosteric modulators in young adults, AU rats would likely be impaired by negative allosteric $GABA_A$ α5 modulation, consistent with recruitment of augmented inhibitory function as a naturally occurring mechanism contributing to resilience in the aging brain. In this context, the present study provides relevant support for further investigation of positive modulators of inhibition to mitigate cognitive decline in aging.

Much has been learned in research on individual differences concerning the alterations that are most closely associated with impairment in neurocognitive aging (Haberman et al., 2017; Leal et al., 2017; Wilson et al., 2006). At the same time, attention directed to the study of resilience in individuals with preserved cognitive function may yield important insights into mechanisms

that contribute to preserved function in the context of aging. Such understanding may point in new directions for therapeutics that optimize healthy brain aging and perhaps mitigate cognitive decline even in the presence of significant brain pathology.

Disclosure

MG is the founder of AgeneBio Incorporated, a biotechnology company that is dedicated to discovery and development of therapies to treat cognitive impairment. She has a financial interest in the company. The authors (MG and RPH) are inventors on Johns Hopkins University's intellectual property that is licensed to AgeneBio. Otherwise, MG has had no consulting relationships with other public or private entities in the past 3 years and has no other financial holdings that could be perceived as constituting a potential conflict of interest. All conflicts of interest are managed by Johns Hopkins University. AB, AM, GB, and JJK have no conflicts of interests to declare.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.neurobiolaging.2018.12.015.

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