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Survey of the *Arc* Epigenetic Landscape in Normal Cognitive Aging

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Abstract

Aging is accompanied by aberrant gene expression that ultimately affects brain plasticity and the capacity to form long-term memories. Immediate-early genes (IEGs) play an active role in these processes. Using a rat model of normal cognitive aging, we found that the expression of *Egr1* and *c-Fos* was associated with chronological age, whereas *Arc* was more tightly linked to cognitive outcomes in aging. More specifically, constitutive *Arc* expression was significantly elevated in aged rats with memory impairment compared to cognitively intact aged rats and young adult animals. Since alterations in the neuroepigenetic mechanisms that gate hippocampal gene expression are also associated with cognitive outcome in aging, we narrowed our focus on examining potential epigenetic mechanisms that may lead to aberrant *Arc* expression. Employing a multilevel analytical approach using bisulfite sequencing, chromatin immunoprecipitations, and micrococcal nuclease digestion, we identified CpG sites in the *Arc* promoter that were coupled to poor cognitive outcomes in aging, histone marks that were similarly coupled to spatial memory deficits, and nucleosome positioning that also varied depending on cognitive status. Together, these findings paint a diverse and complex picture of the *Arc* epigenetic landscape in cognitive aging and bolster a body of work, indicating that dysfunctional epigenetic regulation is associated with memory impairment in the aged brain.

Keywords

Immediate-early gene; Nucleosome positioning; Methylation; Histone modifications; Memory; Plasticity

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Introduction

Vulnerability and resilience to cognitive decline in aging varies greatly from person to person, and the neurobiological underpinnings of cognitive aging across this spectrum are only beginning to be unraveled. Both human and animal models point to altered brain plasticity and connectivity as key determinants of successful versus unsuccessful outcomes in cognitive aging [1, 2]. A major focus of research in this context has been to identify the changes in activity-induced neuronal gene expression that are coupled to cognitive outcomes in aging (e.g., Blalock et al. [3], Haberman et al. [4], and Ianov et al. [5]). Several of those studies implicate the dysregulation of immediate-early gene (IEG) expression, including *Arc*, *Egr-1*, and *c-Fos* that are normally rapidly and selectively upregulated in hippocampal neuron ensembles that are involved in the encoding and recall of long-term memory [6]. However, the mechanisms underlying altered IEG expression in cognitive aging remain unclear.

Regulation of gene expression that supports synaptic plasticity and memory formation involves a complex interplay of transcription factors, cofactors, chromatin regulators, epigenetics, and the core transcription machinery, including its own regulators [7]. Dysfunctional epigenetic mechanisms are particularly notable among these levels of regulation in relation to cognitive outcomes in aged populations [8, 9]. In fact, epigenetic alterations, including changes in DNA methylation and histone modifications (e.g., acetylation, methylation, phosphorylation), are considered a cellular and molecular hallmark of aging [10]. DNA methylation of cytosine residues at CpG dinucleotides is well recognized to correlate with gene silencing, while histone modifications alter chromatin structure in modification-specific, diverse ways. Accessibility to DNA is further regulated at a higher hierarchical level by disrupting, assembling, or moving nucleosomes—a process known as nucleosome remodeling [11]. Together, DNA methylation, histone modifications, nucleosome remodeling, and other epigenetic mechanisms confer a powerful, dynamic, and complex means to fine-tune gene expression in both a temporal-specific and cell type-specific manner.

Although both epigenetics and IEG expression are coupled to cognitive outcome in aging, the impact of the former on the latter is unclear. We therefore took a multilevel approach to examine the epigenetic landscape of IEGs that are associated with age-related spatial memory capacity. Using a well-established model of normal cognitive aging, we first quantified basal and behavior-induced IEG expression levels. We found that *Arc* was more closely related to age-related cognitive outcome than other IEGs, where constitutive *Arc* expression was significantly elevated in aged rats with memory impairments compared to cognitively intact young or aged rats. A closer examination of *Arc* epigenetic regulation in the same animal model uncovered a multifarious epigenetic landscape, where some marks were unique to memory-impaired aged rats, others that associated with intact cognition in aging, and others specific for young animals. While previous research demonstrated that age-related memory impairment is coupled to changes in *Arc* translation and degradation [12], the current study extends our knowledge of *Arc* regulation in cognitive aging at multiple epigenetic levels. The findings are a starting point for pursuing a

more comprehensive account, aimed at identifying the mechanistic links between altered epigenetic regulation and variability in cognitive trajectories across the lifespan.

Material and Methods

Behavioral Characterization

Young (Y; $n = 18$; approximately 6 months of age) and aged ($n = 36$; approximately 24 months of age) male Long-Evans rats (Charles River Laboratories) were housed singly in a climate-controlled vivarium on a 12-h:12-h light:dark cycle with food and water provided ad libitum. Spatial learning and memory were assessed using a hippocampus-dependent, “place” version of the Morris water maze, following an established protocol identical to many previous studies [13–15]. Briefly, key features of the protocol include sparse training (3 trials/day for 8 consecutive days) and the use of multiple, interpolated probe trials (last trial every other day) to document the development of spatial bias for the escape location. Individual differences in learning and memory were assessed according to a learning index (LI) score validated in earlier studies [13, 14], reflecting average proximity to the hidden escape platform over the course of training. By this measure, low scores reflect relatively greater search accuracy focused on the escape location. Aged animals with learning index scores approximating the range of young animals were classified as aged unimpaired (AU, $n = 18$), and those that scored above that range were classified as aged impaired (AI, $n = 18$). These animals were then distributed among three main experiments: gene expression/methylation analyses ($n = 18$), histone modification analyses ($n = 12$), and nucleosome positioning analyses ($n = 24$), as detailed below. Some experiments were exploratory in nature, with insufficient statistical power to detect small age-related differences between groups.

Behavioral Induction of IEGs

In order to examine behavior-induced *Arc* expression, approximately 1 week after behavioral characterization, rats explored a circular environment (Fig. 1b; diameter = 1.8 m) for 5 min (Y, $n = 3$; AU, $n = 3$; AI, $n = 3$). Given that the effects of behavioral IEG induction in this model have been documented in multiple earlier studies [12], the current analyses in comparatively small samples were intended to confirm IEG induction as well as to provide material for methylation analyses. The exploration arena was surrounded by white curtains with patterns affixed, providing spatial cues of the sort used in background behavioral characterization. Animals were euthanized 30 min after the start of exploration, corresponding with the well-documented timepoint of peak *Arc*, *c-Fos*, and *Egr1* gene expression [16]. Although other IEGs (*Homer1a* and *Narp/Nptx2*) were also included, it should be noted that peak expression of these genes is much later, hours after the timepoint examined here. Baseline IEG expression was also measured across Y, AU, and AI rats. These animals were held in the same room as rats undergoing exploratory behavior and were euthanized directly from the home cage (Y, $n = 3$; AU, $n = 3$; AI, $n = 3$). Animals were rapidly anesthetized with 5% isoflurane and decapitated, and brains were extracted. The cerebellum was removed, and the remainder of the brain was separated into two hemispheres. Tissue overlying the medial side of the hippocampus was removed, and using forceps to secure the cerebral cortex, the hippocampus was rolled out and separated from the

remaining tissue. The CA1, CA3, and dentate gyrus (DG) subregions were microdissected in ice-cold phosphate-buffered saline (PBS) under a stereoscope as previously described [17]. Briefly, the DG was pinched out using forceps and the remaining hippocampus was cut along the CA1–CA3 border. Samples were frozen at -80°C until further processing. One hemisphere was used to assess messenger RNA (mRNA) levels, and the other half was used to quantify methylation levels.

Real-Time Quantitative Polymerase Chain Reaction

RNAqueous-4PCR Total RNA Isolation kits (Thermo Fisher Scientific) were used to isolate mRNA from hippocampal tissue using the manufacturer's protocol. Total RNA was then quantified on a NanoDrop 2000 spectrophotometer, and 100 ng was used to generate complementary DNA (cDNA) with iScript Select cDNA Synthesis kits (Bio-Rad). Real-time quantitative polymerase chain reaction (RT-qPCR) was carried out using an Applied Biosystems StepOnePlus Real-Time PCR System Thermal Cycling Block in 96-well plates. Each PCR mixture (20 μl total) contained 1 μl cDNA, 250 mM of each primer (Table 1), 4 μl molecular grade distilled water, and 10 μl iTaq Universal SYBR Green Supermix (Bio-Rad). All reactions were done in triplicate, and serial dilutions of cDNA samples were performed to determine amplification efficiency for each primer pair. The RT-qPCR profile used was 95°C for 3 min followed by 40 cycles of 95°C for 10 s, 55°C for 30 s, and 72°C for 30 s. Following RT-qPCR, melt curve analysis was performed to check for the presence of unwanted products and contaminants in the PCR reaction. The $2^{-\text{Ct}}$ method was employed to analyze the relative changes in gene expression from real-time quantitative PCR experiments, and *Gapdh* was used for normalization.

Bisulfite Sequencing

To explore the possibility that altered CpG methylation of the *Arc* gene might account for dysregulated *Arc* expression in cognitive aging, we quantified methylation levels by bisulfite sequencing. Such modifications can alter transcription by modulating the affinity of DNA-binding proteins [18]. Here, we examined *Arc* methylation under both basal conditions and following activity at CpG sites in three *Arc* regulatory regions: (1) the minimal/proximal *Arc* promoter, (2) an intragenic region, and (3) the synaptic activity-responsive element (SARE), located ~ 7 kb upstream of the rat *Arc* transcription initiation site [19]. This regulatory region has been shown to be necessary for the rapid temporal dynamics of activity-induced *Arc* expression.

Since we observed that hippocampal CA3 *Arc* expression was most tightly coupled to spatial memory impairment in the current experiments, we focused on this region in subsequent analyses. Additional support for focusing on CA3 comes from Haberman et al. [4], who reported that, among the principal cell fields of the hippocampus, gene expression profiles in CA3 are most closely coupled to cognitive status in this model. DNA was isolated from tissue described above using a Qiagen DNeasy Blood & Tissue Kit according to the manufacturer's protocol, and DNA of high quality was ensured by analyzing samples on a Fragment Analyzer (Agilent). Samples were bisulfite converted using an EZ DNA Methylation-Gold Kit (Zymo Research; 1 $\mu\text{g}/\text{sample}$), which results in $> 99\%$ conversion efficiency. Three *Arc* regulatory regions were then amplified with unique sets of primers

(Table 1) containing 8 N random hexamer molecular tags at the 5' ends. PCR products were quality controlled using a Fragment Analyzer. Libraries for each sample were prepared using a NEXTFlex Rapid DNA Kit (PerkinElmer Applied Genomics), and again quality controlled with a Fragment Analyzer. Methyl-Seq library preps and next-generation sequencing of each library was performed with PE250 reads on the MiSeq platform, yielding > 10 K reads per sample.

Adaptor sequences and bases below the Phred score of 33 were removed using the Cutadapt program [20]. Files were then aligned against the rat (rn5) genome using Bowtie 2 [21], and methylation sites were identified using the Bismark program (v0.19) [22].

Chromatin Immunoprecipitation

Histone proteins are composed of a central globular domain and N-terminal tails that contain many modifiable sites including acetylation, methylation, and phosphorylation. As with DNA methylation, these modifications can be potent regulators of gene transcription by modulating access of transcription factors or RNA polymerase to DNA and are important regulators in the processes underlying memory formation and consolidation [23]. We focused on four histone marks that served as marks for various states, which are described below.

In order to examine whether differences in histone modifications might play a role in the elevated levels of constitutive *Arc* expression in unsuccessful cognitive aging, we performed chromatin immunoprecipitations (ChIPs) on the CA3 region of animals euthanized directly from their home cages (Y, $n = 4$; AU, $n = 4$; AI, $n = 4$). Microdissections were performed as described above in ice-cold PBS under a stereoscope, and samples were frozen at -80°C until further processing. Tissue was cut into approximately 4 mm^3 pieces, crosslinked with 1% formaldehyde for 10 min at 37°C , quenched with glycine for 5 min, and washed twice with PBS. Cells were lysed with cell lysis buffer (10 mM Tris (pH 8.0), 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP-40, 0.25% Triton X-100, and protease inhibitor cocktail) using a Dounce homogenizer, and nuclei were collected via centrifugation at 2000g for 5 min at 4°C . Nuclei were lysed with nucleus lysis buffer (10 mM Tris (pH 8.0), 1 mM EDTA, 0.5 mM EGTA, 0.2% SDS, and protease inhibitor cocktail) using a Dounce homogenizer. Chromatin was sheared using a Diagenode Bioruptor XL sonicator on high-power 30-s on, 90-s off cycles for 30 min. Samples were then centrifuged at 15,800g for 10 min at 4°C to remove insoluble debris. A 1% input was set aside for later processing.

The rest of the homogenate was split into five aliquots, and each was diluted with 900 μl dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris (pH 8.1), 167 mM NaCl, and protease inhibitor cocktail). Aliquots were incubated overnight at 4°C with the following ChIP-grade antibodies for histone marks: (1) H3K9Ac (active gene transcription; Cell Signaling no. 9649), (2) H3K9Me2 (inactive gene transcription; Active Motif 39239), (3) H4K12Ac (associated with impaired learning and memory in aged mice; Millipore 07-595), (4) H3K9acS10p (responsive to synaptic activity; Abcam ab12181), or an IgG control (normal rabbit; Santa Cruz sc-2027). Protein-DNA complexes were then captured with 60 μl Protein G Agarose on a rotator for 1 h at 4°C and washed with 1 ml of each of the following buffers for 5 min each: (1) low-salt immune complex wash buffer

(0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris (pH 8.1), 150 mM NaCl), (2) high-salt immune complex wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris (pH 8.1), 500 mM NaCl), and (3) LiCl immune complex wash buffer (0.25 M LiCl, 1% IGEPAL CA630, 1% deoxycholic acid, 1 mM EDTA, 10 mM Tris (pH 8.1)) and twice with TE buffer (10 mM Tris HCl (pH 8.0), 1 mM EDTA).

Chromatin was eluted from the input sample by incubating with 200 μ l elution buffer (100 mM NaHCO₃, 1% SDS) for 30 min. Chromatin was eluted twice from the other samples by adding 100 μ l elution buffer and periodically gently flicking the tube for 15 min. All 200- μ l samples then underwent crosslink reversal by adding 8 μ l of 5 M NaCl and were incubated in a water bath at 65 °C overnight. The samples were incubated for an additional 30 min at 37 °C with 1 μ l RNase A and 2 h at 45 °C with 4 μ l of 0.5 M EDTA, 8 μ l of 1 M Tris HCl, and 1 μ l proteinase K. Chromatin was purified using PCR Clean-Up Kit columns (Mo Bio) and eluted with 50 μ l elution buffer.

RT-qPCR was performed as described above using the primers listed in Table 1. ChIP normalization was carried out using the “% input” method [24].

Nucleosome Positioning

Lastly, we studied *Arc* epigenetics at the higher hierarchical level of nucleosome positioning to assess its potential role in altered expression in cognitive aging. Regulation of gene expression through nucleosome positioning is a dynamic process that differs between genes [25] and is reportedly destabilized in aging, at least in replicative senescence [26].

Approximately 1 week after behavioral characterization, animals were anesthetized with 5% isoflurane and decapitated, and hippocampal subregions were microdissected in aCSF (Tocris) at 4 °C using a dissecting microscope (Y, $n = 8$; AU, $n = 8$; AI, $n = 8$). The CA3 hippocampal subregion was homogenized in 1 ml ice-cold hypotonic buffer (10 mM HEPES, 1.5 mM MgCl₂, 10 mM KCl, and 1 $\times$ Protease and Phosphatase Inhibitor (Pierce)). The homogenate was incubated on ice for 30 min and then centrifuged at 1000 g at 4 °C for 15 min. The supernatant (cytosolic fraction) was removed, and the nuclear pellet was resuspended in hypotonic buffer tubes and centrifuged at 1000 g at 4 °C for 15 min. The supernatant was removed, and the nuclear pellet was used immediately for micrococcal nuclease digestion. Nuclei were warmed to 37 °C, and 1 μ l MNase (50 U/ μ l) was added to 300 μ l nuclei and mixed by pipetting. The reaction was stopped by adding 5 μ l of 250 mM EDTA. To break the nuclear membrane, 5 μ l of 10% SDS was added and the volume was brought up to 0.4 ml. To help dissociate histones and other proteins from DNA, 40 μ l of 5 M NaCl was added. Phenol/chloroform was used to extract, and then with chloroform alone. RNA was removed by incubating with RNase for 30 min at 37 °C. Precipitation took place by adding 1/10 volume sodium acetate (pH 5.2), followed by ethanol precipitation. The pellet was resuspended in 20 μ l TE buffer.

Samples (50 ng each) were used to generate sequencing libraries using the Illumina ChIP-Seq library generation kit. In short, the ends of the sheared DNA fragments were first repaired using a mix of DNA polymerases and ligases, then purified using Agencourt AMPure XP magnetic beads. The 3' ends were adenylated, and Illumina-specific indexed

adaptors were ligated to both ends. These ligation products were purified and size selected to between 300 and 450 bp using a 4.5% agarose gel. The isolated libraries were enriched for fragments containing ligated adaptors on both ends using 18 cycles of PCR followed by a final cleanup with magnetic beads. Sequencing was performed on an Illumina GA II Sequencer using all 8 lanes and run for 42 cycles.

Adaptor sequences and bases below the Phred score of 30 were removed using the Cutadapt program [20]. Files were then aligned against the rat (rn4) genome with very strict parameters using Bowtie 2 [21]. Duplicate reads were removed using Picard (<http://broadinstitute.github.io/picard/>), and the seven replicates were merged into one file for increased sequenced depth, with a minimum width of 80 bases and a maximum width of 250 bases. Nucleosome positions were then identified using nucleosome positioning from sequencing (NPS) software (v2010) [27]. The genome coverage for each region (Online Resource Table 10) was obtained using v2.19 Bedtools [28] and binned into bins of ten bases.

Statistical Analyses

For RT-qPCR and ChIP data, parametric statistics (2-way ANOVAs) were used to compare levels between groups (Y, AU, AI) and conditions (baseline vs exploration). One-way ANOVAs were employed to compare average methylation between groups (Y, AU, AI), while Welch's *t* tests were used for comparisons between control and exploration. Multiple 2-way ANOVAs were applied to compare methylation levels for each CpG site between groups (Y, AU, AI) and between base pairs. Tukey's multiple comparison tests were conducted when the overall group ANOVA reached significance. To compare methylation levels for each CpG site between conditions (cage control/exploratory behavior), multiple *t* tests were employed and corrections for multiple comparisons were carried out using the Holm-Sidak method. For nucleosome positioning data, Kolmogorov-Smirnov tests were used to test for differences between Y, AU, and AI groups for each gene of interest.

Results

Spatial Memory Capacity Varies Substantially Among Aged Individuals

Consistent with previous studies using this model, we observed a wide range of individual differences in hippocampus-dependent spatial learning capacities among aged rats (Fig. 1a). More specifically, while young rats (6 months) scored on average 187 ± 29.4 (SD; Fig. 1, green circles), aged rats varied substantially more with an average of 239 ± 50.7 (Fig. 1, gray squares). Aged animals that scored on par with young animals (< 240) were considered aged unimpaired (AU), while those that performed over that cutoff were considered aged impaired (AI). Successful performance in a single session of non-spatial cue 1 day after the last testing day ensured that no rats with impaired sensorimotor function or motivation were included in the study.

Behavior-Induced IEG Expression Is Blunted in Aged Rats with Memory Impairments

To examine the integrity of activity-dependent IEG expression in relation to age-related cognitive status determined above, we measured mRNA levels 30 min after the start of a

5-min session of exploratory behavior. Despite apparent numerical differences in the percent of time spent in the center or the edge and the number of line crossings, these differences were not statistically significant (all p values > 0.05 ; Fig. 1c). Importantly, since these parameters did not differ between AU and AI rats (p values > 0.05), any downstream effects between these groups cannot be attributed to differences in spatial exploration.

As expected, neither *Homer1a* nor *Narp/Nptx2* showed significant upregulation of expression since the timepoint examined here was optimized for peak expression of other IEGs (Fig. 2; all p values > 0.05). In addition, RT-qPCR analyses revealed that constitutive expression of *Homer1a* or *Narp/Nptx2* does not significantly differ depending on cognitive status (Fig. 2; all p values > 0.05). In contrast, exploratory behavior resulted in significantly elevated *Arc*, *Egr-1*, and *c-Fos* mRNA levels compared to cage controls in all hippocampal subregions examined (Fig. 2, gray significance bars; all p values < 0.05 ; all p values are listed in Online Resource Table 1), post hoc analyses revealed that this increase was largely driven by Y and AU animals (Fig. 2, red significance bars; individual p values are listed in Online Resource Table 1). Indeed, we observed statistically significant interactions between conditions (cage controls/exploratory behavior), cognitive status (Y/AU/AI), and mRNA expression in all hippocampal subregions, with the exceptions of *Egr1* in CA1 and *Arc* in DG (p values are listed in Online Resource Table 2). Further analyses showed significant main group effects between Y, AU, and AI groups (Fig. 2; all p values < 0.05 ; all p values are listed in Online Resource Table 3), and post hoc analyses revealed that this coupling was a mix of both age and cognitive status effects. More specifically, *Arc* expression in AI animals was significantly elevated above Y and AU animals under basal conditions (cage control). Conversely, *Egr1* and *c-Fos* expression levels were generally lower among aged subjects compared to Y following exploratory behavior (Fig. 2, black significance bars; individual p values are listed in Online Resource Table 3). Together, these findings suggest that basal *Arc* gene expression is altered in relation to cognitive aging outcome, while *Egr1* or *c-Fos* gene expression is altered in relation to chronological age.

Basal *Arc* Methylation Is Elevated in Aged Rats with Memory Impairments

DNA methylation plays an important role in regulating the dynamics of gene expression involved in stabilizing and storing long-term memories [29, 30], and changes in DNA methylation over the lifespan are linked to age-related cognitive impairment [31]. To determine whether increased basal *Arc* mRNA expression in cognitively impaired animals is related to changes in DNA methylation, we examined methylation levels at CpG dinucleotide sites in three *Arc* regulatory regions. We focused our analyses on the CA3 region of the hippocampus based on our observation in the previous experiment that cognitive aging outcomes corresponded most tightly with basal *Arc* gene expression in the CA3 region. Additional support for focusing on CA3 comes from Haberman et al. [4], who report that this subfield features the most prominent gene transcription profile coupled to cognitive status in this model.

Exploration had little effect on average methylation levels across regulatory regions. The only significant difference in methylation levels after exploration was observed in the minimal promoter in AI rats (Fig. 3a; $df = 48.12$, $p = 0.01$; individual p values are listed

in Online Resource Table 4). Group comparisons (Y/AU/AI) of average methylation levels for each region under basal conditions and following exploratory behavior showed that the minimal promoter was significantly more methylated in AI compared to Y and AU rats (Fig. 3a; $q = 4.281$ ($p = 0.009$) and $q = 3.896$ ($p = 0.02$), respectively; individual p values are listed in Online Resource Table 5). We then examined methylation at individual bases within each regulatory region because the degree of methylation varied significantly between CpG sites (all p values < 0.05 ; p values are listed in Online Resource Table 6). Post hoc tests identified one CpG site that was significantly more methylated in AI rats compared to AU (115,815,961 bp; $p = 0.0435$), and one site that was significantly more methylated in AI rats compared to Y (115,815,955 bp; $p = 0.0419$; individual p values are listed in Online Resource Table 7). We also found that only 1 bp, located in SARE of the AU group, was differentially methylated in CA3 in response to exploratory behavior (115,822,602 bp; $p = 0.044$; all individual p values are listed in Online Resource Table 8).

Arc-Associated H3K9Me2 and H3K9AcS10p Histone Marks Are Enriched in Aged Rats with Memory Impairment

As with DNA methylation, post-translational modification of histone tails is associated with memory formation [32]. Likewise, altered post-translation modification of histone tails has been linked to aging and has been suggested as a possible mechanism underlying age-related cognitive decline [33]. Here, we quantified basal levels of four different histone modifications in the promoter regions of *c-Fos*, *Erg1*, *Gapdh*, and *Arc* and in the SARE of the *Arc* gene.

Acetylation of histone 3 at lysine 9, H3K9Ac, is a widely used marker of active gene transcription [34]. We hypothesized that elevated levels of basal *Arc* expression in AI rats might parallel this modification. However, group analyses (Y/AU/AI) did not display any significant differences in H3K9Ac enrichment at the *Arc* or *Gapdh* promoters or at SARE (Fig. 4; all p values > 0.05 ; Online Resource Table 9). The frequency distribution of ChIP data is reflected by the width of violin plots in the associated figures.

In contrast with H3K9Ac, dimethylation of histone 3 at lysine 9, H3K9Me2, has been linked to transcriptional repression [35]. No group differences in H3K9Me2 enrichment were evident in SARE or the *Gapdh* promoter (Fig. 4; p values are listed in Online Resource Table 9). However, we observed a significant group effect on enrichment of H3K9Me2 in the proximal *Arc* promoter (Fig. 4; $F_{(2,9)} = 8.321$; $p = 0.009$). Post hoc comparisons identified that under basal conditions, H3K9Me2 is significantly more enriched at the *Arc* promoter in AI in comparison to Y rats (Fig. 4; $p = 0.0079$; all other p values > 0.05 ; Online Resource Table 9). We speculate that this enrichment may represent an unsuccessful compensatory mechanism to dampen the rampant constitutive *Arc* expression observed here in AI rats.

Acetylation of histone 4 at lysine 12, H4K12Ac, is enriched in the promoter and transcribed regions of active genes [34], and *Arc* expression levels correlate with this modification [36]. An earlier study showed similar basal levels of hippocampal H4K12Ac in young and aged mice [33]. In line with this study, we did not observe any significant group effects for any regulatory region examined (Fig. 4; all p values > 0.05 ; Online Resource Table 9).

Lastly, we quantified levels of histone 3 acetylated at lysine 9 and phosphorylated at serine 10, H3K9AcS10p, which was previously shown in vitro to be robustly upregulated at the *Arc* transcription start site 10 min after synaptic activity [37]. As predicted for H3K9Ac, we hypothesized that elevated levels of basal *Arc* expression in AI rats might be associated with enrichment of histones with these modifications in the *Arc* promoter. Indeed, we found that H3K9AcS10p levels in the *Arc* promoter differed between groups (Fig. 4; $F_{(2,9)} = 10.42$; $p = 0.0045$; Online Resource Table 9). Specifically, post hoc comparisons revealed that basal levels of H3K9AcS10p were significantly higher in AI rats than AU rats (Fig. 4; $p = 0.0035$; Online Resource Table 9). We also observed an intermediate value for Y animals under basal conditions. Together, these findings suggest that lower H3K9AcS10p in cognitively intact aged animals may reflect an important compensatory or neuroadaptive mechanism to keep constitutive *Arc* expression in check.

We also quantified levels of the four marks examined above in the *c-Fos* and *Egr1* promoters, which showed no significant differences between groups (Y/AU/AI) for any mark (all p values > 0.05 ; Online Resource Table 10). These findings support the notion that *Arc* is more tightly regulated in relation to cognitive outcome in aging than other IEGs.

Nucleosome Positioning

Loss of histones and higher “fuzziness” in nucleosome positioning has been observed in aging [38], but its functional impact on cognitive aging is unclear. Although our approach using MNase digestion followed by sequencing offered a genome-wide approach, we limited our analysis here to regulatory regions of the IEGs *c-Fos*, *Homer1a*, *Narp/Nptx2*, *Egr1*, and *Arc*. We found that nucleosome positioning surrounding *Arc* regulatory regions significantly differed in aged animals compared to young (Fig. 5; Y vs AU, $p = 0.00016$; Y vs AI, $p = 0.017$) but did not differ based on age-related cognitive status (AU vs AI, $p = 0.12$). Performing these analyses on individual regulatory regions revealed a slightly different profile, where SARE nucleosome positioning only differed between young and aged unimpaired rats (Y vs AU, $p = 0.023$; AU vs AI, $p = 0.38$; Y vs AI, $p = 0.66$). At the *Arc* minimal promoter, a significant difference was observed based on age-related cognitive status (Y vs AU, $p = 0.41$; AU vs AI, $p = 0.0086$; Y vs AI, $p = 0.41$). In the *Arc* intragenic region, however, no significant differences were observed (Y vs AU, $p = 0.082$; AU vs AI, $p = 0.33$; Y vs AI, $p = 0.82$). While no significant differences were observed for *c-Fos*, *Homer1a*, or *Narp/Nptx2*, *Egr1* nucleosome positioning greatly differed in AU animals (Y vs AU, $p = 0.0003$; AU vs AI, $p = 0.00007$; Y vs AI, $p = 0.51$; Online Resource Table 11).

Discussion

Epigenetic regulation of activity-induced gene transcription plays an important role in coordinating the processes that underlie synaptic plasticity and the formation of long-term memories. While altered epigenetic dynamics are an established feature of advanced age [10], their impact on age-related cognitive outcome is unclear. In the current study, we found that behavior-induced *Arc*, *Egr1*, and *c-Fos* expression generally followed a pattern in which AI rats failed to induce expression above basal levels. This was in contrast to AU rats, which, like Y rats, were able to induce IEG expression above basal levels. We also

observed that constitutive expression of *Egr1* and *c-Fos* was more closely associated with chronological age, whereas *Arc* was distinguished as more tightly linked with individual differences in cognitive outcome. We therefore focused our study on the epigenetic mechanisms that might underlie the relationship between dysregulation of *Arc* expression and the spectrum of cognitive aging.

Rapid transcription of *Arc* in the hippocampus is required for long-term memory formation [39, 40]. In agreement with an earlier study [12], we observed promiscuous constitutive *Arc* expression among aged rats with spatial memory deficits. Importantly, following exploratory behavior, aged impaired animals failed to induce the rapid *Arc* transcription that supports hippocampus-dependent learning and memory. While Fletcher et al. [12] identified multiple post-transcriptional mechanisms linked to aberrant *Arc* expression, the current study offers valuable insight into the *Arc* epigenomic transcriptional gatekeeping capacity that distinguishes impaired and successful cognitive aging. Our integrative approach to characterizing the *Arc* epigenetic landscape in cognitive aging depicts a multiplex scene. Across levels of analysis—nucleosome positioning, histone modifications, and DNA methylation—we observed that some aspects of *Arc* epigenetics remain intact regardless of age or cognitive outcome, while others appear compensatory or neuroadaptive, with the potential to maintain optimal *Arc* regulation.

An earlier study examining *Arc* methylation in aging reported differences in methylation in Fischer 344 rats that depended on age (9–12 months vs 24–32 months), experience (basal vs spatial behavior), and hippocampal subregion (CA1 vs DG) [41]. We extend those findings in part by focusing on the CA3 subregion, which, in our model, shows maximal basal *Arc* dysregulation (Fig. 2) and a broader profile of gene transcriptional change coupled to age-related cognitive status [4]. Similar to Penner et al. [41], which reported significantly elevated CA1 basal methylation of *Arc* at the promoter region among aged animals, we observed that aged rats with memory impairment had significantly higher levels of methylation than either aged rats with intact memory or young rats. Despite documented changes in methylation with age in the prefrontal cortex and hippocampus (e.g., [31, 42]), the direction of effect from gene to gene is highly heterogeneous [43] and highlights the importance of examining methylation at the base level. We identified a pair of individual CpG sites in the *Arc* minimal promoter at which methylation was significantly elevated under basal conditions in cognitively impaired aged rats compared to young adults and aged unimpaired rats. Some evidence suggests that the methylation status of even a single CpG site can have consequential effects on gene transcription [31, 44] and by extension, on memory.

Another novel advance from previous work is the inclusion of SARE—an enhancer region that induces a synaptic activity-induced transcriptional response [19]. Since a previous study reported substantial changes in *Arc* methylation in CA1 and DG at the minimal promoter and intragenic region following spatial exploration [41], we were surprised to find that only 1 bp, located in SARE of the AU group, was differentially methylated in response to exploratory behavior. Although Penner et al. [41] reported behavior-induced CA1 demethylation of the minimal promoter across young and aged animals, the response was more robust among aged rats. Another behavior-induced change in methylation was a

significant overall demethylation of the minimal promoter in CA3 in aged impaired rats. It is possible that the timepoint examined was suboptimal to capture the full extent of temporal dynamics of behavior-induced (de)methylation. In line with this possibility, scarce activity-induced changes in *Arc* methylation and generally low *Arc* methylation levels, like those observed here, have been reported elsewhere [45]. In that study, electroconvulsive stimulation induced changes in methylation that were not observed until 24 h after stimulation. Based on the current data, we speculate that the behavior-induced demethylation is driven by cognitively impaired individuals as an unsuccessful compensatory mechanism to bring *Arc* expression above baseline levels.

Like DNA methylation, histone modifications associated with memory in advanced age depend on recent behavioral experience, chronological aging, cognitive status, and brain region [46]. In regard to histone acetylation, one study reported a decrease in hippocampal *Arc* expression with chronological age (2-month-, 7-month-, and 18-month-old mice) that was coupled to a concomitant increase in *Arc* promoter-associated histone deacetylase 2 (HDAC2) and a decrease in H3K9 acetylation. In the aged mice with memory impairments, treatment with HDAC2 antisense or by the HDAC inhibitor (HDACi) sodium butyrate restored memory function [47]. Findings from the current study more closely align with another study that showed an absence of significant differences in H3K9 acetylation with respect to age or cognitive status [46]. Although HDACis have garnered interest as potential pharmacological interventions to reverse cognitive impairment [48], other evidence calls into question the potential utility of systemic, relatively non-specific HDAC inhibition as a therapeutic strategy for promoting healthy memory function in aging [49].

Together with histone acetylation, histone methylation also regulates gene expression important for synaptic plasticity and memory formation. For example, dimethylation of H3K9 (a repressive marker of transcription) is reduced 24 h after fear conditioning, thereby relieving transcriptional repression [50]. As with DNA methylation in the current study, we observed that basal H3K9Me2 levels are also enriched in aged animals with memory impairments. This modification might therefore be another compensatory attempt to curb aberrant *Arc* expression in cognitively impaired aged rats. Following synaptic activity, H3K9Me2 is actively demethylated concurrent with an increase in H3K9AcS10P—a histone mark closely associated with activity [37]. Here, we observed that cognitively impaired aged animals, compared with the aged hippocampus in animals with intact memory, are enriched in H3K9AcS10P. Since this modification is tightly associated with synaptic activity, the current findings bolster several studies that report hippocampal hyperexcitability among aged animals and humans with memory deficits (e.g., [51, 52]). H3K9AcS10P is particularly interesting, as levels of this mark in the young adult CA3 were intermediate between the aged cognitive phenotypes. This unique profile among aged unimpaired rats may therefore represent a neuroadaptive mechanism whereby H3K9AcS10P supports an optimally healthy outcome in cognitive aging [2].

Nucleosome positioning is crucial for accurately regulating gene expression [53], and this organization appears to be disturbed in aging [26, 38]. The current study offers an initial analysis, beyond replicative senescence in yeast [26], into whether the canonical organization of nucleosomes is intact in terminally differentiated and highly heterogeneous

mammalian brain cells. Such studies will be important to establish the degree to which changes in nucleosome organization contribute to reported changes in transcriptional profiles in aging. Examination of nucleosome positioning in the rat model used here also allowed us to probe whether these changes are coupled with cognitive outcomes in aging. Indeed, our exploratory data suggest that positioning of nucleosomes varies depending on age-related cognitive status and this association is gene specific. The extent to which these differences are linked to altered gene expression and the direction of effect remain to be explored. As computational challenges are overcome [54] and approaches to address cell heterogeneity improve [55], it will be important to more fully examine the influence nucleosome positioning has on cognitive outcome in aging.

Together, our results identify neuroepigenetic mechanisms that are coupled to dysregulated *Arc* expression in age-related cognitive decline. An important future direction in this line of work will be to establish the degree to which the epigenetic differences observed here are the primary determinants of disrupted IEG expression in cognitive aging. While a major strength of the study was analysis at multiple levels, a more exhaustive account of the epigenetic landscape in cognitive aging should also include additional epigenetic mechanisms such as microRNAs, long non-coding RNAs, hydroxymethylation, and other histone modifications. A consideration of other genes, additional brain regions implicated in cognitive aging, cell type specificity, and a more thorough characterization of epigenetic temporal dynamics and how they are affected by aging will also be critical. Although in its infancy, epigenetic editing represents a promising gene-specific method to modulate gene expression [56]. Applied to *Arc*, these technologies could lead to novel therapeutics to rectify dysregulated *Arc* expression in unsuccessful cognitive aging as well other diseases where *Arc* is associated with impaired cognition.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Morrison JH, Baxter MG (2012) The ageing cortical synapse: hallmarks and implications for cognitive decline. *Nat Rev Neurosci* 13:240–250. 10.1038/nrn3200 [PubMed: 22395804]
2. Rapp PR, Bañuelos C, Myrum C (2020) Neuroadaptive trajectories of healthy mindspan: from genes to neural networks. In: Thomas AK, Gutchess A (eds) *Handbook of cognitive aging*. Cambridge University Press
3. Blalock EM, Chen K-C, Sharrow K, Herman JP, Porter NM, Foster TC, Landfield PW (2003) Gene microarrays in hippocampal aging: statistical profiling identifies novel processes correlated with cognitive impairment. *J Neurosci* 23:3807–3819. 10.1523/JNEUROSCI.23-09-03807.2003 [PubMed: 12736351]

4. Haberman RP, Colantuoni C, Stocker AM, Schmidt AC, Pedersen JT, Gallagher M (2011) Prominent hippocampal CA3 gene expression profile in neurocognitive aging. *Neurobiol Aging* 32: 1678–1692. 10.1016/j.neurobiolaging.2009.10.005 [PubMed: 19913943]
5. Ianov L, DeBoth M, Chawla MK et al. (2017a) Hippocampal transcriptomic profiles: subfield vulnerability to age and cognitive impairment. *Front Aging Neurosci* 9:1–15. 10.3389/fnagi.2017.00383 [PubMed: 28174533]
6. Minatohara K, Akiyoshi M, Okuno H (2016) Role of immediate-early genes in synaptic plasticity and neuronal ensembles underlying the memory trace. *Front Mol Neurosci* 8:1–11. 10.3389/fnmol.2015.00078
7. Alberini CM, Kandel ER (2015) The regulation of transcription in memory consolidation. *Cold Spring Harb Perspect Biol* 7:a021741. 10.1101/cshperspect.a021741
8. Spiegel AM, Sewal AS, Rapp PR (2014) Epigenetic contributions to cognitive aging: disentangling mindspan and lifespan. *Learn Mem* 21:569–574. 10.1101/lm.033506.113 [PubMed: 25227252]
9. Barter JD, Foster TC (2018) Aging in the brain: new roles of epigenetics in cognitive decline. *Neurosci* 24:516–525. 10.1177/1073858418780971
10. López-Otín C, Blasco MA, Partridge L, Serrano M, Kroemer G (2013) The hallmarks of aging. *Cell* 153:1194–1217. 10.1016/j.cell.2013.05.039 [PubMed: 23746838]
11. Becker PB, Workman JL (2013) Nucleosome remodeling and epigenetics. *Cold Spring Harb Perspect Biol* 5:a017905–a017905. 10.1101/cshperspect.a017905 [PubMed: 24003213]
12. Fletcher BR, Hill GS, Long JM, Gallagher M, Shapiro ML, Rapp PR (2014) A fine balance: regulation of hippocampal Arc/Arg3.1 transcription, translation and degradation in a rat model of normal cognitive aging. *Neurobiol Learn Mem* 115:58–67. 10.1016/j.nlm.2014.08.007 [PubMed: 25151943]
13. Gallagher M, Burwell R, Burchinal M (1993) Severity of spatial learning impairment in aging: development of a learning index for performance in the Morris water maze. *Behav Neurosci* 107:618–626. 10.1037/0735-7044.107.4.618 [PubMed: 8397866]
14. Gallagher M, Burwell R, Burchinal M (2015) Severity of spatial learning impairment in aging: development of a learning index for performance in the Morris water maze. *Behav Neurosci* 129:540–548. 10.1037/bne0000080 [PubMed: 26214219]
15. Myrum C, Rossi SL, Perez EJ, Rapp PR (2019) Cortical network dynamics are coupled with cognitive aging in rats. *Hippocampus*. 10.1002/hipo.23130
16. Guzowski JF, Setlow B, Wagner EK, McGaugh JL (2001) Experience-dependent gene expression in the rat hippocampus after spatial learning: a comparison of the immediate-early genes Arc, c-fos, and zif268. *J Neurosci* 21:5089–5098. 10.1523/JNEUROSCI.21-14-05089.2001 [PubMed: 11438584]
17. Myrum C, Rapp PR (2019) Isolation and quantification brain region-specific and cell subtype-specific histone (de)acetylation in cognitive neuroepigenetics. In: *Methods in molecular biology: protein acetylation*. Springer Nature, pp. 265–277
18. Heyward FD, Sweatt JD (2015) DNA methylation in memory formation: emerging insights. *Neuroscientist* 21:475–489. 10.1177/1073858415579635 [PubMed: 25832671]
19. Kawashima T, Okuno H, Nonaka M, Adachi-Morishima A, Kyo N, Okamura M, Takemoto-Kimura S, Worley PF et al. (2009) Synaptic activity-responsive element in the Arc/Arg3.1 promoter essential for synapse-to-nucleus signaling in activated neurons. *Proc Natl Acad Sci* 106:316–321. 10.1073/pnas.0806518106 [PubMed: 19116276]
20. Martin M (2011) Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.journal* 17:10. 10.14806/ej.17.1.200
21. Langmead B, Salzberg SL (2012) Fast gapped-read alignment with Bowtie 2. *Nat Methods* 9:357–359. 10.1038/nmeth.1923 [PubMed: 22388286]
22. Krueger F, Andrews SR (2011) Bismark: a flexible aligner and methylation caller for Bisulfite-Seq applications. *Bioinformatics* 27:1571–1572. 10.1093/bioinformatics/btr167 [PubMed: 21493656]
23. Day JJ, Sweatt JD (2011) Epigenetic mechanisms in cognition. *Neuron* 70:813–829. 10.1016/j.neuron.2011.05.019 [PubMed: 21658577]

24. Haring M, Offermann S, Danker T, Horst I, Peterhansel C, Stam M (2007) Chromatin immunoprecipitation: optimization, quantitative analysis and data normalization. *Plant Methods* 3:11. 10.1186/1746-4811-3-11 [PubMed: 17892552]
25. Struhl K, Segal E (2013) Determinants of nucleosome positioning. *Nat Struct Mol Biol* 20:267–273. 10.1038/nsmb.2506 [PubMed: 23463311]
26. Hu Z, Chen K, Xia Z, Chavez M, Pal S, Seol JH, Chen CC, Li W et al. (2014) Nucleosome loss leads to global transcriptional upregulation and genomic instability during yeast aging. *Genes Dev* 28:396–408. 10.1101/gad.233221.113 [PubMed: 24532716]
27. Zhang Y, Shin H, Song JS, Lei Y, Liu XS (2008) Identifying positioned nucleosomes with epigenetic marks in human from ChIP-Seq. *BMC Genomics* 9:537. 10.1186/1471-2164-9-537 [PubMed: 19014516]
28. Quinlan AR, Hall IM (2010) BEDTools: a flexible suite of utilities for comparing genomic features. *Bioinformatics* 26:841–842. 10.1093/bioinformatics/btq033 [PubMed: 20110278]
29. Halder R, Hennion M, Vidal RO, Shomroni O, Rahman RU, Rajput A, Centeno TP, van Bebber F et al. (2016) DNA methylation changes in plasticity genes accompany the formation and maintenance of memory. *Nat Neurosci* 19:102–110. 10.1038/nn.4194 [PubMed: 26656643]
30. Sweatt JD (2016) Dynamic DNA methylation controls glutamate receptor trafficking and synaptic scaling. *J Neurochem* 137:312–330. 10.1111/jnc.13564 [PubMed: 26849493]
31. Ianov L, Riva A, Kumar A, Foster TC (2017b) DNA methylation of synaptic genes in the prefrontal cortex is associated with aging and age-related cognitive impairment. *Front Aging Neurosci* 9:249. 10.3389/fnagi.2017.00249 [PubMed: 28824413]
32. Peixoto L, Abel T (2013) The role of histone acetylation in memory formation and cognitive impairments. *Neuropsychopharmacology* 38:62–76. 10.1038/npp.2012.86 [PubMed: 22669172]
33. Peleg S, Sananbenesi F, Zovoilis A, Burkhardt S, Bahari-Javan S, Agis-Balboa RC, Cota P, Wittmann J et al. (2010) Altered histone acetylation is associated with age-dependent memory impairment in mice. *Science* 328:753–756. 10.1126/science.1186088 [PubMed: 20448184]
34. Wang Z, Zang C, Rosenfeld JA, Schones DE, Barski A, Cuddapah S, Cui K, Roh T et al. (2008) Combinatorial patterns of histone acetylations and methylations in the human genome. *Nat Genet* 40:897–903. 10.1038/ng.154 [PubMed: 18552846]
35. Black JC, Van Rechem C, Whetstone JR (2012) Histone lysine methylation dynamics: establishment, regulation, and biological impact. *Mol Cell* 48:491–507. 10.1016/j.molcel.2012.11.006 [PubMed: 23200123]
36. Wee CL, Teo S, Oey NE et al. (2014) Nuclear Arc interacts with the histone acetyltransferase Tip60 to modify H4K12 acetylation. *Eneuro* 1. 10.1523/ENEURO.0019-14.2014
37. Oey NE, Leung HW, Ezhilarasan R et al. (2015) A neuronal activity-dependent dual function chromatin-modifying complex regulates Arc expression. *eNeuro* 2. 10.1523/ENEURO.0020-14.2015
38. Song S, Johnson F (2018) Epigenetic mechanisms impacting aging: a focus on histone levels and telomeres. *Genes (Basel)* 9:201. 10.3390/genes9040201
39. Guzowski JF, Lyford GL, Stevenson GD, Houston FP, McGaugh J, Worley PF, Barnes CA (2000) Inhibition of activity-dependent arc protein expression in the rat hippocampus impairs the maintenance of long-term potentiation and the consolidation of long-term memory. *J Neurosci* 20:3993–4001. 10.1523/jneurosci.20-11-03993.2000 [PubMed: 10818134]
40. Plath N, Ohana O, Dammermann B, Errington ML, Schmitz D, Gross C, Mao X, Engelsberg A et al. (2006) Arc/Arg3.1 is essential for the consolidation of synaptic plasticity and memories. *Neuron* 52:437–444. 10.1016/j.neuron.2006.08.024 [PubMed: 17088210]
41. Penner MR, Roth TL, Chawla MK, Hoang LT, Roth ED, Lubin FD, Sweatt JD, Worley PF et al. (2011) Age-related changes in Arc transcription and DNA methylation within the hippocampus. *Neurobiol Aging* 32:2198–2210. 10.1016/j.neurobiolaging.2010.01.009 [PubMed: 20189687]
42. Masser DR, Hadad N, Porter HL, Mangold CA, Unnikrishnan A, Ford MM, Giles CB, Georgescu C et al. (2017) Sexually divergent DNA methylation patterns with hippocampal aging. *Aging Cell* 16:1342–1352. 10.1111/ace.12681 [PubMed: 28948711]
43. Hadad N, Masser DR, Logan S, Wronowski B, Mangold CA, Clark N, Otolara L, Unnikrishnan A et al. (2016) Absence of genomic hypomethylation or regulation of cytosine-modifying enzymes

- with aging in male and female mice. *Epigenetics Chromatin* 9:30. 10.1186/s13072-016-0080-6 [PubMed: 27413395]
44. Sohn BH, Park IY, Lee J et al. (2010) Functional switching of TGF- β 1 signaling in liver cancer via epigenetic modulation of a single CpG site in TTP promoter. *Gastroenterology* 138:1898–1908.e12. 10.1053/j.gastro.2009.12.044 [PubMed: 20038433]
 45. Dyrvig M, Hansen HH, Christiansen SH, Woldbye DP, Mikkelsen JD, Lichota J (2012) Epigenetic regulation of Arc and c-Fos in the hippocampus after acute electroconvulsive stimulation in the rat. *Brain Res Bull* 88:507–513. 10.1016/j.brainresbull.2012.05.004 [PubMed: 22613772]
 46. Castellano JF, Fletcher BR, Kelley-Bell B et al. (2012) Age-related memory impairment is associated with disrupted multivariate epigenetic coordination in the hippocampus. *PLoS One* 7:e33249. 10.1371/journal.pone.0033249 [PubMed: 22438904]
 47. Singh P, Thakur MK (2018) Histone deacetylase 2 inhibition attenuates downregulation of hippocampal plasticity gene expression during aging. *Mol Neurobiol* 55:2432–2442. 10.1007/s12035-017-0490-x [PubMed: 28364391]
 48. Gräff J, Tsai L-H (2013) Histone acetylation: molecular mnemonics on the chromatin. *Nat Rev Neurosci* 14:97–111. 10.1038/nrn3427 [PubMed: 23324667]
 49. Castellano JF, Fletcher BR, Patzke H, Long JM, Sewal A, Kim DH, Kelley-Bell B, Rapp PR (2014) Reassessing the effects of histone deacetylase inhibitors on hippocampal memory and cognitive aging. *Hippocampus* 24:1006–1016. 10.1002/hipo.22286 [PubMed: 24753063]
 50. Gupta S, Kim SY, Artis S, Molfese DL, Schumacher A, Sweatt JD, Paylor RE, Lubin FD (2010) Histone methylation regulates memory formation. *J Neurosci* 30:3589–3599. 10.1523/JNEUROSCI.3732-09.2010 [PubMed: 20219993]
 51. Yassa MA, Lacy JW, Stark SM, et al. (2010) Pattern separation deficits associated with increased hippocampal CA3 and dentate gyrus activity in nondemented older adults. *Hippocampus* n/a–n/a. doi: 10.1002/hipo.20808
 52. Haberman RP, Koh MT, Gallagher M (2017) Heightened cortical excitability in aged rodents with memory impairment. *Neurobiol Aging* 54:144–151. 10.1016/j.neurobiolaging.2016.12.021 [PubMed: 28104309]
 53. Schones DE, Cui K, Cuddapah S, Roh TY, Barski A, Wang Z, Wei G, Zhao K (2008) Dynamic regulation of nucleosome positioning in the human genome. *Cell* 132:887–898. 10.1016/j.cell.2008.02.022 [PubMed: 18329373]
 54. Flores O, Deniz Ö, Soler-López M, Orozco M (2014) Fuzziness and noise in nucleosomal architecture. *Nucleic Acids Res* 42:4934–4946. 10.1093/nar/gku165 [PubMed: 24586063]
 55. Lai B, Gao W, Cui K, Xie W, Tang Q, Jin W, Hu G, Ni B et al. (2018) Principles of nucleosome organization revealed by single-cell micrococcal nuclease sequencing. *Nature* 562:281–285. 10.1038/s41586-018-0567-3 [PubMed: 30258225]
 56. DeGroote ML, Verschure PJ, Rots MG (2012) Epigenetic editing: targeted rewriting of epigenetic marks to modulate expression of selected target genes. *Nucleic Acids Res* 40:10596–10613. 10.1093/nar/gks863 [PubMed: 23002135]

Fig. 1.

Spatial learning capacity assessment and exploratory behavior in rats used to examine IEG expression and epigenetics. **a** Individual animal learning index scores derived from a standardized test of spatial learning in the water maze. These rats were distributed among multiple subsequent experiments. **b** Circular arena (diameter = 1.8 m) where rats freely explored for 5 min. **c** Percent of time that rats spent in either the edge or center and/or the number of times rats crossed any line was quantified. Individual animals are displayed as black dots

Fig. 2.

Arc, *Egr1*, *c-Fos*, *Homer1a*, and *Narp/Nptx2* mRNA levels in CA1, CA3, and dentate gyrus (DG) in Y, AU, and AI rats at baseline or 30 min after exploratory behavior. Means (+ SEM) are expressed as a percentage of young baseline values. * $p < 0.05$; ** $p < 0.001$; *** $p < 0.0001$; **** $p < 0.00001$

Fig. 3. Methylation levels at three *Arc* regulatory regions in CA3 at both baseline and 30 min after exploratory behavior in Y, AU, and AI rats. **a** Average methylation over respective *Arc* regulatory regions. **b** Percent methylation at the base level. * $p < 0.05$; ** $p < 0.001$

Fig. 4.

Violin plots of *Arc*-associated histone marks in CA3 at baseline in Y, AU, and AI rats. Enrichment of four histone marks (H3K9Ac, H3K9me2, H4K12Ac, and H3K9AcS10p) at the *Gapdh* and *Arc* promoter, and *Arc*'s enhancer region, SARE, was determined using ChIP followed by RT-qPCR. Rabbit IgG was used as a negative control and values are presented as % of input. The width of plots reflects the frequency distribution of the data. * $p < 0.05$

Fig. 5.

Nucleosome positioning of *Arc* regulatory regions in CA3 at baseline in Y, AU, and AI rats. MNase digestion was carried out followed by sequencing. Each peak represents a single nucleosome. The top panel displays the *Arc* coding region, plus ~ 7 kb upstream (Y vs AU, $p = 0.00016$; AU vs AI, $p = 0.12$; Y vs AI, $p = 0.017$). The bottom panels displays *Arc* regulatory regions: SARE (Y vs AU, $p = 0.023$; AU vs AI, $p = 0.38$; Y vs AI, $p = 0.66$), the *Arc* minimal promoter (Y vs AU, $p = 0.41$; AU vs AI, $p = 0.0086$; Y vs AI, $p = 0.41$), and the *Arc* intragenic region (Y vs AU, $p = 0.082$; AU vs AI, $p = 0.33$; Y vs AI, $p = 0.82$). Shaded areas indicate SARE, the minimal promoter, and the intragenic regions, respectively. Plotted data were binned in 10-bp intervals

Primers used in the current study

Table 1

Primer	Sequence	Application
<i>Arc</i> F	CCCAGTCTGTGGCTTTTGTCA	mRNA/RT-qPCR
<i>Arc</i> R	GTGTCAGCCCCAGCTCAATC	mRNA/RT-qPCR
<i>c-Fos</i> F	ACGGAGAAATCCGAAGGGAAAGGAA	mRNA/RT-qPCR
<i>c-Fos</i> R	TCTGCAACGCAGACTTCTCGTCTT	mRNA/RT-qPCR
<i>Egr1</i> F	TCTAGTGTGAAGGGAGCAA	mRNA/RT-qPCR
<i>Egr1</i> R	ACTTTCAGCTGCCCTGAAACAG.	mRNA/RT-qPCR
<i>Gapdh</i> F	AATGGGAGTTGCTGTTGAAG	mRNA/RT-qPCR
<i>Gapdh</i> R	CTGGAGAAACCTGCCAAGTA	mRNA/RT-qPCR
<i>Homer1a</i> F	TGATAGCCGGGCAACACT	mRNA/RT-qPCR
<i>Homer1a</i> R	TCCTCTCTGCTGATTCTCTGTG	mRNA/RT-qPCR
<i>Narp</i> F	GGCAAGATCAAGAAGACGTTG	mRNA/RT-qPCR
<i>Narp</i> R	TCCAGGTGATGCAGATATGTT	mRNA/RT-qPCR
<i>Arc</i> promoter F	ACGGCCCCCGCGCAGCATAAAA	ChIP/RT-qPCR
<i>Arc</i> promoter R	TTAGCGAGTGTAGCAGGCTCGCC	ChIP/RT-qPCR
<i>Arc</i> SARE F	TCTGCGCGTAGAGCCTTC	ChIP/RT-qPCR
<i>Arc</i> SARE R	TTTCTCTGCTGTTCTCTGCAA	ChIP/RT-qPCR
<i>c-Fos</i> promoter F	AACGACCCCTTCAGGCATCC	ChIP/RT-qPCR
<i>c-Fos</i> promoter R	GTTTAAAGGACGACAGCAC	ChIP/RT-qPCR
<i>Egr1</i> promoter F	GCGCCACCGCTCTTGGAT	ChIP/RT-qPCR
<i>Egr1</i> promoter R	CGAATCGGCCTCTATTCAA	ChIP/RT-qPCR
<i>Gapdh</i> promoter F	ACCATGCTTCACTGACATTCTGA	ChIP/RT-qPCR
<i>Gapdh</i> promoter R	GGTCTGCCTCCCTGCTAACC	ChIP/RT-qPCR
<i>Arc</i> minimal promoter F	GGTTAATGGGAGTTAGGGTTT	Bisulfite/sequencing
<i>Arc</i> minimal promoter R	TCAATAAATCCAACCTCCATCTACTC	Bisulfite/sequencing
<i>Arc</i> SARE F	TTTTTTTATAGGTAGGGGTAGAGTAG	Bisulfite/sequencing
<i>Arc</i> SARE R	ATTTTCTACTATTCTCTACAATTCATTTA	Bisulfite/sequencing
Intragenic <i>Arc</i> F	TGTGATTTTGTAGATTGTAAGTGT	Bisulfite/sequencing
Intragenic <i>Arc</i> R	CAACCTTAATAAATCTCTTCCAAC	Bisulfite/sequencing