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A fine balance: Regulation of hippocampal Arc/Arg3.1 transcription, translation and degradation in a rat model of normal cognitive aging

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ABSTRACT

Memory decline is a common feature of aging. Expression of the immediate-early gene *Arc* is necessary for normal long-term memory, and although experience dependent *Arc* transcription is reportedly reduced in the aged rat hippocampus, it has not been clear whether this effect is an invariant consequence of growing older, or a finding linked specifically to age-related memory impairment. Here we show that experience dependent *Arc* mRNA expression in the hippocampus fails selectively among aged rats with spatial memory deficits. While these findings are consistent with the possibility that blunted *Arc* transcription contributes to cognitive aging, we also found increased basal ARC protein levels in the CA1 field of the hippocampus in aged rats with memory impairment, together with a loss of the experience dependent increase observed in young and unimpaired aged rats. Follow-up analysis revealed that increased basal translation and blunted ubiquitin mediated degradation may contribute to increased basal ARC protein levels noted in memory impaired aged rats. These findings indicate that *Arc* expression is regulated at multiple levels, and that several of these mechanisms are altered in cognitively impaired aged rats. Defining the influence of these alterations on the spatial and temporal fidelity of synapse specific, memory-related plasticity in the aged hippocampus is an important challenge.

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1. Introduction

Many individuals develop cognitive deficits during normal aging, prominently involving memory, while others at the same chronological age perform on par with younger adults. In humans and animal models, this loss of function can occur in the context of largely preserved neuron and synapse numbers throughout the hippocampal memory system, and impairment is thought to arise instead from subtle alterations in the plasticity and connectivity required to form and maintain memories (Burke & Barnes, 2006; Fletcher, 2012).

Expression of the immediate early gene *Arc* (activity-regulated cytoskeleton-associated protein; also termed Arg3.1) is necessary for multiple forms of synaptic plasticity, and is induced under a variety of behavioral and experimental conditions, including LTP (Lyford et al., 1995; Steward, Wallace, Lyford, & Worley, 1998), LTD, novel environmental exploration (Guzowski, McNaughton, Barnes, & Worley, 1999; Pinaud, Penner, Robertson, & Currie, 2001; Vazdarjanova, 2004), and learning (Fletcher, 2006; Guzowski, Setlow, Wagner, & McGaugh, 2001; Pinaud et al., 2001). Blocking *Arc* protein expression by anti-sense oligonucleotide injection impairs LTP maintenance and memory consolidation while sparing short-term memory, suggesting a potentially critical role in stabilizing enduring synaptic modifications (Guzowski et al., 2000). ARC protein also regulates synaptic strength and homeostatic scaling by promoting the internalization of AMPARs (Chowdhury et al., 2006; Rial Verde, Lee-Osbourne, Worley, Malinow, & Cline, 2006; Shepherd et al., 2006). This background suggests the possibility that disrupted *Arc* induction or processing might give rise to impairment in memory-related hippocampal plasticity associated with aging. Recent studies have reported data consistent with that proposal, prompting the conclusion that changes in *Arc* transcription, mediated in part by epigenetic regulation, may contribute to impoverished consolidation and poor memory retrieval in aging (Burke, Ryan, & Barnes, 2012; Marrone, Satvat, Shaner, Worley, & Barnes, 2012; Ménard & Quirion, 2012; Penner et al., 2011; Blalock et al., 2003).

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The transcription, translation, trafficking and turnover of *Arc* mRNA and protein are tightly regulated and vary according to cell and stimulus type. Within minutes of NMDA receptor (NMDAR) or voltage-gated calcium channel activation, a burst of *Arc* transcription is initiated in all principal fields of the hippocampus (Adams, Robinson, Hudgins, Wissink, & Dudek, 2009), lasting for hours in the dentate gyrus (Lyford et al., 1995), and subsiding more rapidly in pyramidal neurons (Guzowski et al., 1999). Resulting mRNA is trafficked and targeted to specific synapses in an NMDAR-dependent manner (Farris, Lewandowski, Cox, & Steward, 2014; Moga et al., 2004; Steward et al., 1998), and although it has been widely thought to target recently activated synapses, current evidence indicates that locally translated ARC preferentially accumulates at relatively less active synaptic sites (Okuno et al., 2012). This account suggests that *Arc* mediates an 'inverse tagging' mechanism, distinguishing potentiated synapses by inhibiting enhancement at weak or inactive sites through AMPA receptor endocytosis. Temporal regulation of *Arc* expression is achieved via a network of activating and repressive factors that control transcription and translation (Bramham et al., 2009). Regulation of mRNA stability by translation dependent decay (TDD) pathways provides an additional means of controlling the dendritic localization of *Arc* mRNA and fine tuning Arc protein levels (Farris et al., 2014; Giorgi et al., 2007). Finally, ARC protein turnover is regulated by ubiquitin proteasome dependent degradation (Greer et al., 2010; Kuehnle, Mothes, Matentzoglu, & Scheffner, 2013). Disrupted ARC protein regulation at the synapse is suspected to play a role in a variety of disorders in which cognitive function is prominently affected, including Angelman Syndrome (Greer et al., 2010), Huntington's disease (Maheshwari, Samanta, Godavarthi, Mukherjee, & Jana, 2012) and fragile-X syndrome (Niere, Wilkerson, & Huber, 2012).

Current evidence indicates that experience dependent Arc transcription is blunted in the aged rat hippocampus, consistent with a potential contribution to the memory impairment that frequently accompanies aging (Penner, Chawla, Roth, Sweatt, & Barnes, 2010). On the basis of available studies, however, it has been difficult to disentangle whether reduced *Arc* transcription is an obligatory consequence of chronological aging, or a finding more specifically tied to the effects of aging on memory supported by the hippocampus. A previous study in Fischer 344 rats, for example, reported that aged subjects exhibit both memory impairment and decreased *Arc* expression in the CA1 field of the hippocampus relative to young controls, but no correlation between the mRNA and cognitive results (Blalock et al., 2003). In addition, earlier work on *Arc* in the context of neurocognitive aging has concentrated predominantly on mRNA expression, and how the complex regulatory network responsible for ARC protein translation might be affected has not been examined. Independent of neurocognitive aging, limited attention has been directed at testing the possibility that, like *Arc* mRNA induction, the regulatory factors that mediate *Arc* translational control may also be dynamically modulated in response to recent behavioral training (Barker-Haliski, Pastuzyn, & Keefe, 2012). As a starting point in filling these gaps, here we quantified experience dependent *Arc* transcription and translation in a rat model that reveals substantial individual differences in cognitive aging, from aged subjects that display significant deficits in spatial learning and memory supported by the hippocampus, to age-matched rats that perform as well as young adults. Overall, the results indicate that changes in the dynamic regulation of hippocampal *Arc* in relation to age-related memory impairment extend beyond transcription, involving multiple levels of control, including both translation and degradation.

2. Methods

2.1. Water maze training

2.1.1. Background behavioral characterization

Young ($n = 36$; 6 months of age) and aged ($n = 71$; approximately 24 months of age) male Long-Evans rats (Charles River Laboratories) were housed singly in a climate-controlled vivarium on a 12:12 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 (Gallagher, Burwell, & Burchinal, 1993). 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 score validated in earlier studies (Gallagher et al., 1993), 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 unimpaired (AU), and those that scored above that range were classified as impaired (AI). These animals were subsequently used to analyze *Arc* transcription and translation, as described in the following sections.

2.1.2. Behavioral induction of Arc transcription

To induce *Arc* transcription rats were trained on a redundant place–cue (RPC) task in a new testing environment (young, $n = 11$; AU, $n = 9$; AI, $n = 9$), two weeks after background behavioral characterization. Training consisted of five days of testing (two, 2-trial blocks per day, 25 min between blocks) throughout which a visible platform was maintained in a constant location. During the last trial on the final day the platform was lowered to the bottom of the tank for the first 30 s of the trial, making it unavailable for escape, after which it was raised and visible. During this probe, young and aged-unimpaired rats demonstrate a significant bias for the former, cued escape location, whereas aged–impaired rats exhibit no evidence of spatial learning. Accordingly, the multi-day redundant place/cue procedure provided a setting sensitive to age-related memory impairment while minimizing nonspecific performance differences across groups that might influence *Arc*. Animals were killed five minutes after the last trial (30 min after the first trial) and tissue was processed for *Arc* in situ hybridization. This time point was chosen on the basis of the well documented time-course of *Arc* mRNA transcription (Guzowski et al., 1999), in order to maximize *Arc* mRNA levels in the CA1 and CA3 regions of the hippocampus. To assess baseline levels of *Arc* mRNA, rats held in the same room as animals that underwent RPC training were killed directly from their home cage (young, $n = 5$; AU, $n = 4$; AI, $n = 5$).

2.1.3. Behavioral induction of Arc translation

In order to examine behaviorally induced *Arc* translation, two weeks after background behavioral characterization, rats (young, $n = 10$; AU, $n = 12$; AI, $n = 10$) were tested on a single session RPC task that consisted of 15 trials with a 15 s inter-trial interval. For trials 1 through 9, and 11, 13, and 15, the escape platform was visible. On the remaining three, interleaved trials (10, 12, and 14), the platform was slightly submerged and hidden from view, but available for escape. The platform remained in a constant location throughout training, and the start location was varied pseudo-randomly across trials.

Two hours after the first training trial, animals were rapidly anesthetized with 5% isoflurane, decapitated, and brains were extracted. The CA1, and CA3 subregions were microdissected in 4 °C aCSF (Tocris Bioscience) under a stereoscope, and samples were frozen at −80 °C until further processing. Baseline protein levels were assessed in the hippocampus from subjects held in the same room as animals that received RPC training, but that were killed directly from their cage (young, $n = 10$; AU, $n = 12$; aged AI, $n = 10$). The two-hour testing to sacrifice interval was chosen based on the known temporal kinetics for the first wave of *Arc* translation induced by experience (Ramirez-Amaya, 2005). We also elected to use a within-day RPC protocol to eliminate any potential contribution to ARC protein levels from behavioral testing on the day prior to sacrifice, reflecting the second wave of *Arc* translation that reportedly occurs at 24 h (Ramirez-Amaya, 2005). It should be noted, however, that massed training in the 1-session version of the RPC task is not optimally designed for characterizing age-related variability in spatial memory capacity (see Section 3). Accordingly, all post-mortem gene and protein expression results were evaluated in relation to measures from standard background training in the water maze, adopting an approach validated in many previous studies (Fletcher & Rapp, 2012).

2.2. Arc in situ hybridization and quantification

Generation of ^{35}S labeled sense and anti-sense probes and hybridization to tissue sections was performed as previously described (Fletcher, 2006). Material from young, AU and AI rats was processed together, enabling direct comparisons of signal intensity, independent of run-to-run variability. Image J software (http://rsb.info.nih.gov/ij/) was used to trace the regions of interest (ROI) from films scanned at 1200 pixels per inch. Mean optical density for each ROI (CA1, CA3 and somatosensory cortex) was averaged across four evenly spaced sections (600 μm interval), beginning at the dorsal pole of the hippocampus for each animal. The intensity of *Arc* mRNA expression in the dentate gyrus was too low to permit accurate regional delineation and quantification.

2.3. Western blotting

2.3.1. Immunoblotting

Cytosolic sample fractions prepared from the microdissected CA3 and CA1 hippocampal subregions (for detailed fractionation methods see, Castellano et al., 2012) were normalized for total protein concentration and separated by SDS–PAGE using 4–12% or 12% Bis–Tris gels (Invitrogen) and MES–SDS or MOPS–SDS Buffer with 1X anti-oxidant (Invitrogen). Primary antibodies were diluted in blocking solution (2% ECL advance blocking agent; GE Healthcare) in wash buffer (TBS with 0.1% Tween-20) and applied to membranes overnight at 4 °C. Immunoreactivity was detected with 0.6 ug/ml Alexa 488, Alexa 633 or Cy3 conjugated secondary antibodies (Jackson ImmunoResearch Laboratories). All groups and conditions were represented within each blot for each hippocampal subfield. Immunoblots were scanned at a resolution of 100 μm/pixel on a Typhoon Trio Plus scanner (GE Healthcare), quantified using ImageQuant TL image analysis software (GE Healthcare), and averaged across duplicate runs. Values for each animal were then normalized to the mean young baseline value for that protein. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was measured on every blot as a loading control, and in no case did GAPDH vary between groups or condition (CA1: main effect of group $F_{(2,58)} = 0.197$, $p = 0.882$, condition $F_{(1,58)} = 2.567$, $p = 0.115$, interaction $F_{(2,58)} = 0.007$, $p = 0.993$; CA3: main effect of group $F_{(2,58)} = 0.604$, $p = 0.550$, condition $F_{(1,58)} = 0.068$, $p = 0.796$, interaction $F_{(2,58)} = 0.213$, $p = 0.809$).

2.3.2. Primary antibodies

The following primary antibodies were used at the indicated dilutions: Arc (1:250, Santa Cruz, C7), eIF4A3 (1:2500, ProteinTech, 17,504-AP), eIF4E (1:1000, Abgent, AM1852a), GAPDH (1:500, Santa Cruz Biotechnology, 25,778), Ube3A (1:500, Abgent, AT4445a), Upf1 (1:500, Millipore, 07-1014). Antibody specificity was confirmed by competition with appropriate peptides (results not shown).

2.4. Statistical analysis

Parametric statistics (MANOVA) were used to compare measures across groups (Young, AU, AI) and conditions (baseline, behaviorally activated). Follow-up comparisons were conducted by either LSD or *t*-tests, as appropriate. Levene's test was used to determine if equal variances were assumed in *t*-test calculations. Potential associations between learning index scores and relevant measures were assessed by Pearson r correlation coefficients.

3. Results

3.1. Effects of aging on spatial learning vary substantially among individuals

Consistent with the results of previous studies in this model (for review see, (Fletcher & Rapp, 2012; Gallagher & Rapp, 1997), we detected substantial individual differences in performance in the standardized version of the Morris water maze among the aged rats used to examine *Arc* transcription (Fig. 1A) and translation (Fig. 1B). Approximately half of the aged rats performed on par with young adults (i.e. aged unimpaired, AU), and the remainder displayed varying degrees of impairment beyond this normative range (i.e. aged impaired, AI). In order to examine behaviorally induced *Arc* translation, two weeks after background behavioral characterization, rats were tested on a 1-session RPC task. All groups performed equivalently across the initial nine cued trials (repeated measures ANOVA trial * group interaction $F_{(16,232)} = 0.924$, $p = 0.543$, Fig. 1C), while aged rats performed significantly worse on hidden platform trials (main effect of group $F_{(2,29)} = 13.832$, $p < 0.001$, Fig. 1D). Aged impaired rats also performed significantly worse on the final three cued trials than young rats (main effect of group $F_{(2,29)} = 5.792$, $p < 0.01$, Fig. 1D). This effect is surprising given that AI rats performed equivalently to young rats on the initial nine cued trials and may result from increasing fatigue and slow swimming speed in the aged impaired rats due to longer swim times on the preceding hidden trials. Nonetheless, learning index scores derived from performance on the standardized version of the water maze task were highly correlated with individual animal performance on hidden trials of the RPC task used to induce *Arc* translation ($r = 0.63$, $p < 0.001$, Fig. 1E), indicating that this procedure provides a reliable assessment of spatial learning capacity. Learning index scores were used in all correlational analyses so that both baseline and activated groups could be compared using the same behavioral measure of spatial learning capacity. Additionally, the learning index comprises a composite score derived from spatial memory performance across multiple days of testing and has been shown to be a more sensitive, stable and accurate measure for identifying individual differences in spatial memory than massed training (Gallagher et al., 1993; Maei, 2009). Neurobiological comparisons in the context of this behavioral characterization can reliably distinguish changes related to chronological age from those underlying age-related memory impairment (reviewed in Fletcher & Rapp, 2012).

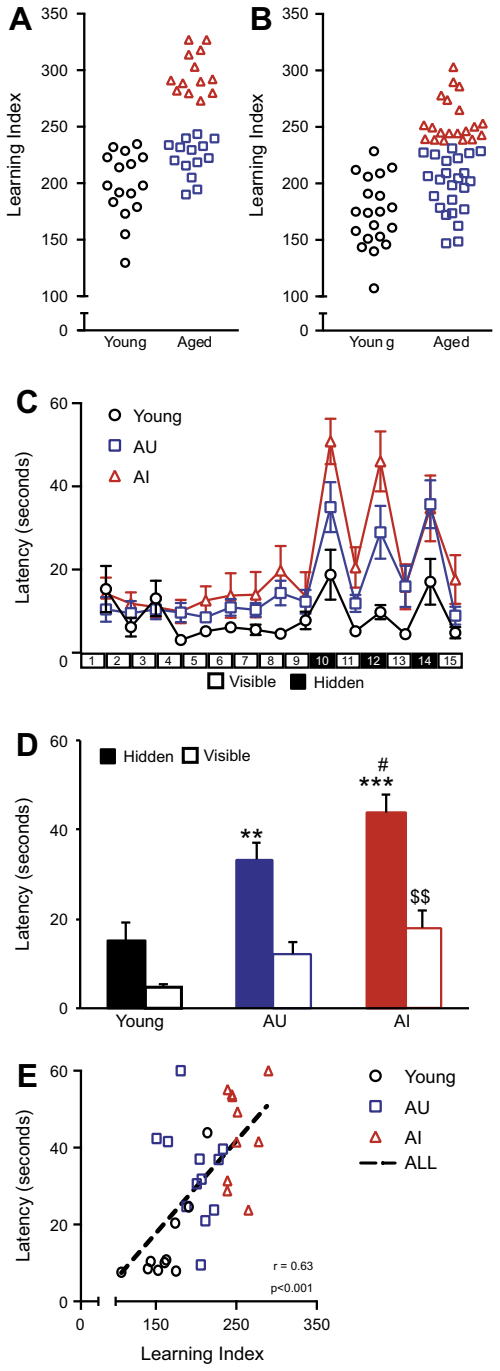

Fig. 1. Spatial learning capacity in rats used to examine *Arc* transcription (A) and translation (B–E). Learning index scores from standard water maze training for individual young (black), aged unimpaired (blue), and aged impaired rats (red) used to examine *Arc* transcription (A) and translation (B). (C) All groups tested on the 1-session RPC task performed well on cued trials (1–9, 11, 13, 15), while AI rats scored poorly on hidden trials (10, 12, 14). (D) Average escape latency (+SEM) on hidden platform trials (filled columns) was shorter in young rats than aged rats ($^{**}p < 0.01$, $^{***}p < 0.001$) and shorter in AU rats than AI rats ($^{\#}p = 0.05$). Average escape latency on visible trials (open columns) was longer in AI rats than in young rats ($^{\$\$}p < 0.01$). (E) Higher learning index scores from standard water maze training correlated with longer average latency to reach the platform on hidden platform trials in the single session RPC procedure.

3.2. Cognitive aging is associated with impaired behavioral regulation of Arc transcription in brain regions critical for spatial memory

Capitalizing on the variability observed in spatial memory among the aged subjects, *Arc* mRNA expression was measured in multiple brain regions in order to determine whether experience dependent transcription is disrupted in relation to the cognitive consequences of aging or chronological age. RPC training was associated with robust increases in *Arc* mRNA transcription relative to baseline cage controls in CA1 (main effect of condition $F_{(1,38)} = 12.597$, $p = 0.001$, Fig. 2A), CA3 (main effect of condition $F_{(1,37)} = 6.411$, $p = 0.016$, Fig. 2B) and somatosensory cortex (main effect of condition $F_{(1,37)} = 37.337$, $p < 0.001$, Fig. 2C). In the hippocampus, however, training dependent increases in *Arc* mRNA were selectively observed in young and aged rats with intact memory (CA1: Y, $t_{(1,14)} = 3.685$, $p = 0.002$; AU, $t_{(1,8)} = 2.372$, $p = 0.05$; AI, $t_{(1,12)} = 0.872$, $p = 0.400$; Fig. 2A, CA3: Y, $t_{(1,14)} = 3.015$, $p = 0.009$; AU, $t_{(1,10)} = 2.397$, $p = 0.037$; AI, $t_{(1,12)} = 0.243$, $p = 0.812$; Fig. 2B). In contrast, young rats and both aged subgroups displayed robust training induced *Arc* expression in the somatosensory cortex (Y, $t_{(1,14)} = 4.116$, $p = 0.001$; AU, $t_{(1,11)} = 3.325$, $p = 0.007$; AI, $t_{(1,12)} = 2.363$, $p = 0.036$; Fig. 2C). The latter findings count against the possibility that aging is associated with a distributed defect in transcriptional integrity. Among the regions that were examined, deficits in experience dependent *Arc* transcription were instead restricted to the hippocampus, i.e., an area where dysfunction is known to contribute to cognitive impairment in animal models and aged humans (for reviews see, Burke & Barnes, 2010; Fletcher & Rapp, 2012; Gallagher, Bakker, Yassa, & Stark, 2010).

3.3. Basal ARC protein levels are elevated in the CA1 field of the aged impaired hippocampus, and fail to increase in response to behavioral training

We next sought to determine if the blunted *Arc* transcription observed in AI rats in response to RPC training results in correspondingly low ARC protein levels. Western blotting was used to quantify protein in CA1 and CA3 of young, AU and AI rats at baseline or two hours after a single session RPC training protocol (Fig. 3).

As expected, ARC protein levels increased significantly as a result of RPC training in CA1 relative to basal home cage control values (main effect of condition: $F_{(1,54)} = 5.089$, $p = 0.028$; Fig. 3A). Post-hoc comparisons, however, revealed that this effect was attributable to reliable increases in young and AU subjects, whereas ARC protein levels in AI rats were insensitive to behavioral training (Y, $t_{(1,18)} = -2.972$, $p = 0.008$; AU, $t_{(1,22)} = -2.034$, $p = 0.054$; AI, $t_{(1,18)} = -0.143$, $p = 0.888$). Further analysis revealed that basal ARC protein levels varied across groups (main effect of group: $F_{(2,30)} = 8.245$, $p = 0.002$), ranking significantly higher in aged impaired rats than young ($p < 0.001$), and equivalent with values for all groups provided RPC training (main effect of group: $F_{(2,27)} = 0.057$, $p = 0.945$). In the CA3 field, by comparison, ARC protein showed no induction with behavioral training (main effect of condition: $F_{(1,56)} = 0.107$, $p = 0.745$; Fig. 3B), but differed across groups (main effect of group: $F_{(2,56)} = 3.186$, $p = 0.049$). Specifically, post hoc comparisons revealed that CA3 ARC protein levels were significantly higher in AU than young rats ($p = 0.014$). Together, these results suggest that the influence of behavioral training on *Arc* transcription in the aged hippocampus are at least partly uncoupled from subsequent ARC protein expression. Consistent with the speculation that disrupted ARC protein regulation impacts memory, elevated ARC levels in CA1 correlated with poor water maze performance (across all subjects for basal ARC protein levels, $r = 0.43$, $p = 0.014$; and for AU rats among those provided RPC training, $r = 0.62$, $p = 0.031$; Fig. 3C and E).

Although discordance in mRNA and protein expression is not uncommon (Mijalski et al., 2005), we expected closer correspondence in the present case because *Arc* mRNA is a target of translation dependent decay (TDD) (Farris et al., 2014; Giorgi et al., 2007). TDD degrades transcripts after a single round of translation,

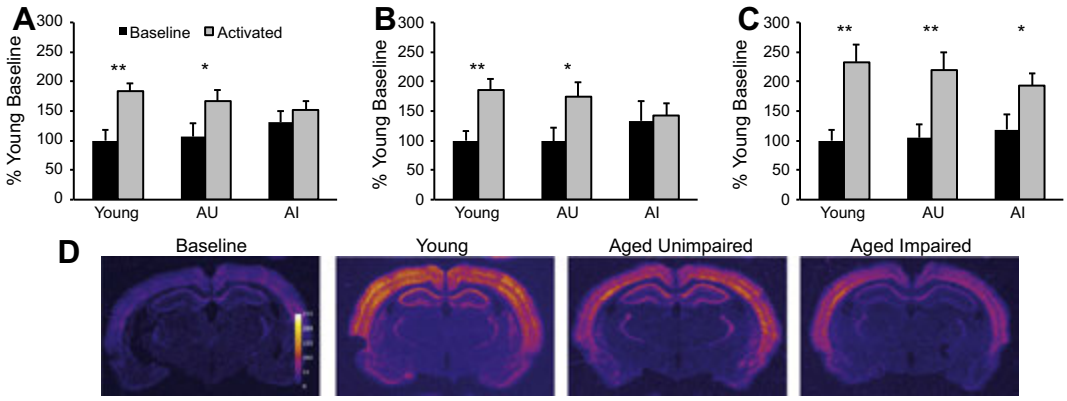

Fig. 2. Aged rats with poor spatial memory fail to induce *Arc* mRNA transcription in CA1 and CA3 following behavioral training. *Arc* mRNA levels were quantified in CA1 (A), CA3 (B), and somatosensory cortex (C) in young, AU and AI rats at baseline or 30 min after RPC training (activated). Means (+SEM) are expressed as a percentage of young baseline values. (D) Pseudo-colored representative autoradiographs of *Arc* mRNA in situ hybridization.

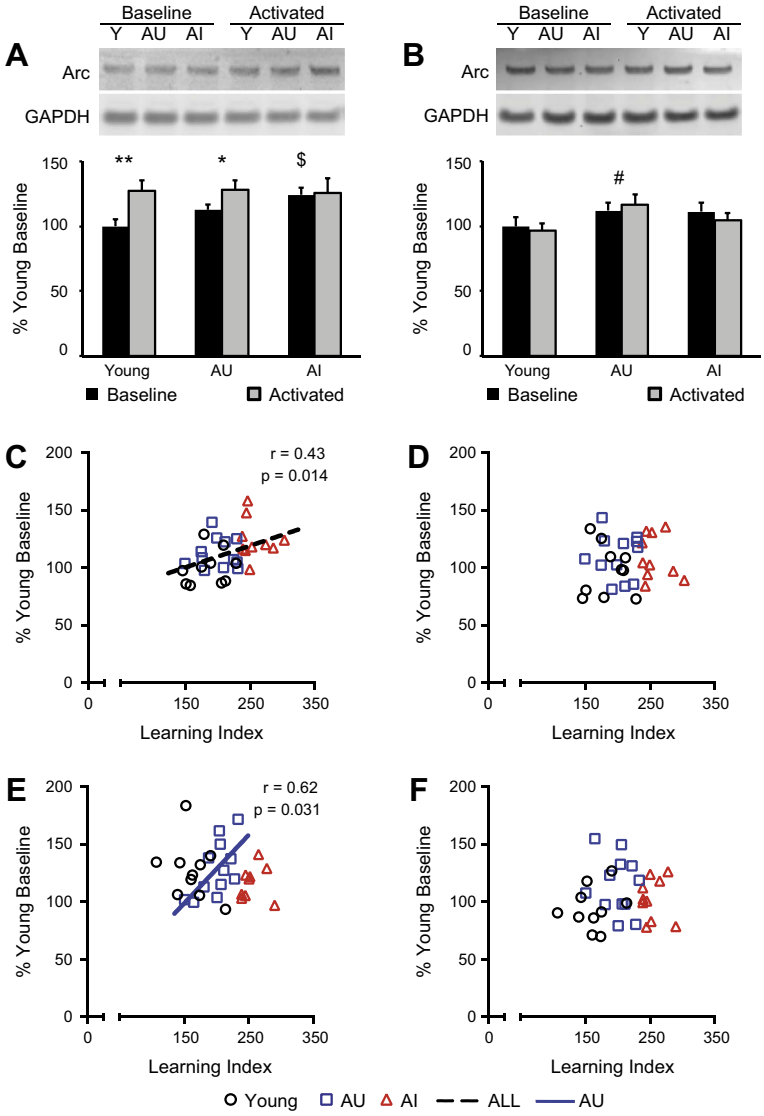

Fig. 3. Basal ARC protein levels are elevated in CA1 in aged impaired rats and unresponsive to RPC training. Representative western blots and quantification of ARC protein levels in CA1 (A) and CA3 (B) in young, AU and AI rats at baseline or 2 h after RPC training (activated). Means (+SEM) are expressed as a percentage of young baseline values ($^{*}p < 0.05$, $^{**}p < 0.01$ activated versus baseline values; $^{\$}p < 0.05$ versus young baseline values; $^{\#}p < 0.05$ versus young). Scatterplots of ARC protein levels in CA1 (C and E) and CA3 (D and F) in young, AU and AI rats at baseline (C and D) or 2 h after RPC training (E and F) plotted against individual subject learning index scores. Significant correlations are denoted by trend lines.

thereby tightly regulating associated protein levels (Maquat, Hwang, Sato, & Tang, 2011). Additionally, ARC protein turns over quickly and thus has a relatively short half-life, in some cases as short as 37 min (Messaoudi et al., 2007; Rao et al., 2006; Soule et al., 2012). It has also been shown that local *Arc* mRNA level is highly predictive of ARC protein level in vivo when induced by either learning or LTP (Farris et al., 2014). In an effort to determine the basis of the discrepancy between mRNA and protein observed in AI rats, we next focused on known regulators of *Arc* translation, stability and degradation. None of the factors examined varied as a function of age, cognitive status or training condition in CA3, and the following description therefore focuses on findings for the CA1 field of the hippocampus.

3.4. Translation dependent decay mechanisms remain largely intact in aged rats

Translation dependent decay serves as a brake on *Arc* translation by allowing only a single round of translation before an mRNA transcript is targeted for degradation (Giorgi et al., 2007). This process is triggered by a stop codon more than 50–55 nucleotides upstream of an exon–exon junction, marked during RNA splicing by deposition of an exon junctional complex (EJC). Eukaryotic initiation factor 4A3 (eIF4A3) is a constitutive mRNA binding component of the EJC that negatively regulates ARC protein levels (Giorgi et al., 2007). In the present experiments eIF4A3 levels in CA1 were equivalent in comparisons between groups and conditions (Fig. 4A). We also examined levels of up frameshift 1 (Upf1), an ATPase that is critical for initiating TDD following the pioneer round of translation (Chang, Imam, & Wilkinson, 2007). Like eIF4A3, Upf1 levels in CA1 were unaffected in relation to group and condition (Fig. 4B). These results suggest that a large-scale disruption of TDD mechanisms is unlikely to account for the constitutively elevated *Arc* translation observed in CA1 in aged animals with memory impairment.

3.5. Regulation is shifted in favor of increased basal Arc translation in the AI hippocampus

Formation of the initiation complex is generally considered the rate-limiting and most tightly regulated step of cap-dependent translation (Costa-Mattioli, Sossin, Klann, & Sonenberg, 2009). The eukaryotic initiation factor 4E (eIF4E) is the least abundant of the initiation complex constituents and is thought to be a limiting factor for translational activity (Gingras, Raught, & Sonenberg, 1999). In the present experiments total eIF4E was regulated following RPC training and in relation to cognitive status in CA1 (main effect of condition: $F_{(1,54)} = 5.353$, $p = 0.025$; group * condition interaction: $F_{(2,54)} = 3.479$, $p = 0.038$; Fig. 5A). Whereas post hoc comparisons confirmed that the training induced increase was reliable or marginally significant in both young and AU rats (Y, $t_{(1,18)} = -3.105$, $p = 0.006$; AU, $t_{(1,22)} = -1.919$, $p = 0.068$), AI subjects failed to display training dependent increases in eIF4E relative to baseline values ($t_{(1,18)} = 0.995$, $p = 0.330$). Similar to the pattern of ARC protein expression, basal eIF4E was elevated in AI rats relative to young controls ($p = 0.032$). Thus, memory impaired aged rats exhibited constitutively elevated ARC and eIF4E protein levels that were unresponsive to behavioral training. ARC protein levels in all groups across both conditions positively correlated with eIF4E levels ($r = 0.40$ $p = 0.001$; Fig. 5C) consistent with the idea that eIF4E is a positive regulator of *Arc* translation or is under the control of the same signaling cascades. Recent studies document that LTP in the dentate gyrus induces phosphorylation of eIF4E, and that inhibiting this effect blunts *Arc* translation (Panja et al., 2009). In contrast to these findings, the ratio of phosphorylated to total eIF4E was not significantly altered in relation to age (main effect of group: $F_{(2,54)} = 0.031$, $p = 0.970$) or condition (main effect of condition: $F_{(1,56)} = 7.871$, $p = 0.079$; Fig. 5B), and was negatively correlated with ARC expression ($r = -0.27$ $p = 0.03$; Fig. 5D). The overall pattern of results suggests that this correlation is likely driven by the change in total eIF4E rather than a substantial task- or age-related change in eIF4E phosphorylation.

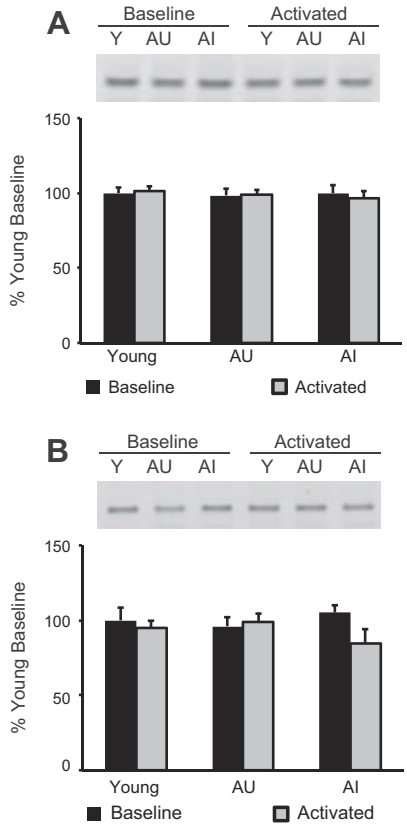

Fig. 4. Levels of eIF4A3 and Upf1 protein in CA1 are unaffected in relation to age and RPC training. Representative western blots and quantification of eIF4A3 (A) and Upf1 (B) protein levels CA1 in young, AU and AI rats at baseline or 2 h after RPC training (activated). Means (+SEM) are expressed as a percentage of young baseline values.

3.6. Ube3a in CA1 is decreased in aged rats

ARC protein levels are also controlled by the E3 ubiquitin ligase, Ube3a, which targets the protein for degradation through ubiquitination (Greer et al., 2010). In agreement with previous studies examining other brain regions (Bhat, Yan, Wang, Li, & Li, 2014; Williams, 2010), we found that Ube3a is decreased in CA1 in the aged hippocampus. Specifically, we found a main effect of group on Ube3a protein ($F_{(2,54)} = 4.037$, $p = 0.023$; Fig. 6A), and post hoc analysis confirmed that levels were significantly lower in AI rats relative to young ($p = 0.010$). Low Ube3a protein levels in CA1 also correlated with poor spatial memory ($r = -0.32$, $p = 0.01$; Fig. 6B). Amplifying the shift toward increased translation, low levels of Ube3a may slow protein degradation, thereby contributing to the ARC protein elevation observed in CA1 in memory impaired aged animals.

4. Discussion

Our findings document that, despite significantly decreased behavioral induction of *Arc* mRNA transcription in aged impaired rats, basal ARC protein levels in CA1 are increased relative to controls and fail to respond to water maze training. A related study demonstrated that recent behavioral experience induces *Arc* tran-

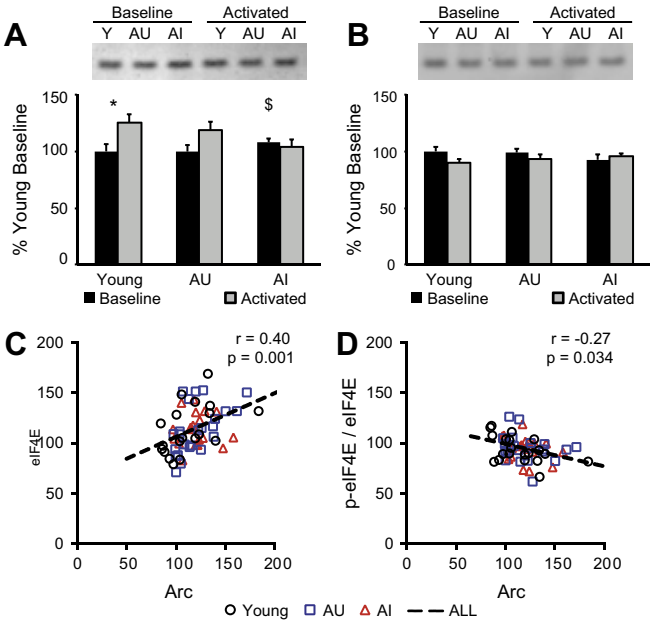

Fig. 5. Basal eIF4E levels are elevated in CA1 in aged impaired rats and unresponsive to RPC training. Representative western blots and quantification of eIF4E (A) and phospho-eIF4e (B) in CA1 in young, AU and AI rats at baseline or 2 h after RPC training (activated). Means (+SEM) are expressed as a percentage of young baseline values ($^{*}p < 0.05$, activated versus baseline values; $^{\$}p < 0.05$ versus young baseline values). Levels of eIF4E protein (as a percentage of young controls) positively correlated with ARC protein in CA1 (C), while the ratio of phosphorylated to total eIF4E inversely correlated with ARC (D). Significant correlations are denoted by trend lines.

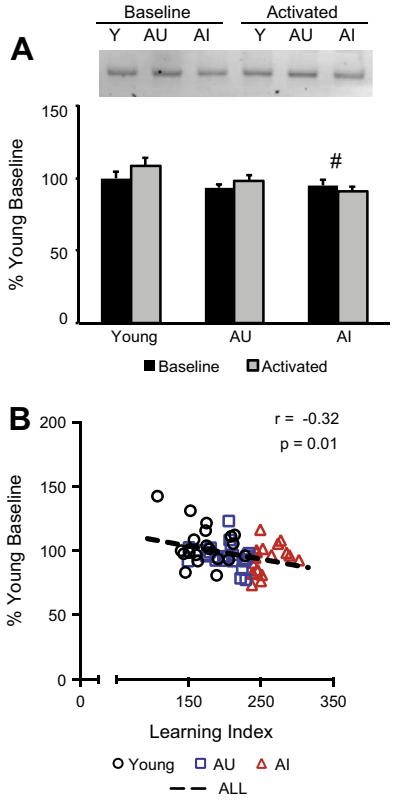

Fig. 6. Ube3a is reduced in CA1 in aged impaired rats. (A) Representative western blots and quantification of Ube3a protein levels in CA1 of young, AU and AI rats at baseline or 2 h after RPC training (activated) ($^{\#}p < 0.05$ versus young). (B) Ube3a levels inversely correlated with spatial learning index scores such that low levels were associated with impairment.

scription in equivalent numbers of neurons in the young and aged hippocampus, but that the amount of *Arc* mRNA expressed per neuron is reduced in aged rats (Penner et al., 2010). Here we confirm and extend those findings, demonstrating that the failed behavioral induction of *Arc* transcription is not an obligatory consequence of chronological aging, and is instead specifically related to variability in the cognitive outcome of aging. In a novel extension we also examined the relationship between *Arc* mRNA and protein levels. As expected for young and aged rats with intact memory that displayed increased *Arc* mRNA levels in hippocampus 30 min after RPC training, ARC protein levels exhibited a corresponding increase in CA1 at 2 h post-training. In aged rats with memory deficits, however, CA1 ARC protein levels were elevated at baseline and failed to increase further in response to RPC training.

Arc mRNA and protein levels are highly correlated under normal conditions. *Arc* mRNA is degraded by TDD mechanisms following only a few rounds of translation (Giorgi et al., 2007), and ARC protein has a relatively short half-life of 37 min (Soule et al., 2012), accounting for the tight correspondence between RNA and protein. Given that behavioral training in aged impaired rats failed to induce the *Arc* transcription observed in the young and AU hippocampus, we expected that ARC protein levels would also be lower in AI rats. Contrary to predictions we found that ARC protein levels in aged impaired rats were both elevated at baseline and insensitive to recent behavioral experience. In order to explore the potential basis of this unexpected result, we examined known regulators of mRNA stability, translation and protein turnover (Fig. 7). The observation that regulators of translation as well as degradation are altered highlights the importance of evaluating the broader regulatory networks that control *Arc*, rather than relying on mRNA transcription as a proxy for protein expression.

Activity dependent translation of mRNA is required for multiple forms of synaptic plasticity as well as learning and memory, and is a highly regulated means of controlling local protein levels. Initiation is the rate-limiting step of cap-dependent translation under most circumstances and a hub for translational control. The formation of the initiation complex is limited by the availability of the RNA binding subunit, eIF4E. In our experiments we found that eIF4E protein levels were increased following RPC training in young and aged rats with intact cognition. We also found that basal eIF4E is increased in aged rats with memory impairment, suggesting that basal cap-dependent translation may be higher in these rats relative to young or aged unimpaired rats. Indeed genetically increased eIF4E activity in mice results in exaggerated cap-dependent translation, as well as social and cognitive deficits (Gkogkas et al., 2013; Santini et al., 2014). Increased eIF4E activity also results in an increased ratio of synaptic excitation to inhibition (Gkogkas et al., 2013; Santini et al., 2014), consistent with the elevated neuronal activity seen in children with autism (Cornew, Roberts, Blaskey, & Edgar, 2011), as well as cognitively impaired aged rats (Wilson, Ikonen, Gallagher, Eichenbaum, & Tanila, 2005; Wilson et al., 2004) and humans (Bakker et al., 2012; Yassa, Stark, & Gallagher, 2010). In the case of age-related memory impairment, elevated neuronal activity in the hippocampus is restricted to the CA3 region, and as might be predicted as a consequence, changes in proteostasis reported here were largely selective for the primary post-synaptic target of this excess excitation, the CA1 field of the hippocampus. A pattern of excess basal protein synthesis and loss of stimulus-induced translation in brain has also been documented in models of fragile X syndrome (Bassell & Warren, 2008; Muddashetty, Kelic, Gross, Xu, & Bassell, 2007). Taken together these data point to elevated basal cap-dependent translation as a possible common factor contributing to cognitive impairment across a variety of otherwise diverse conditions including normal aging.

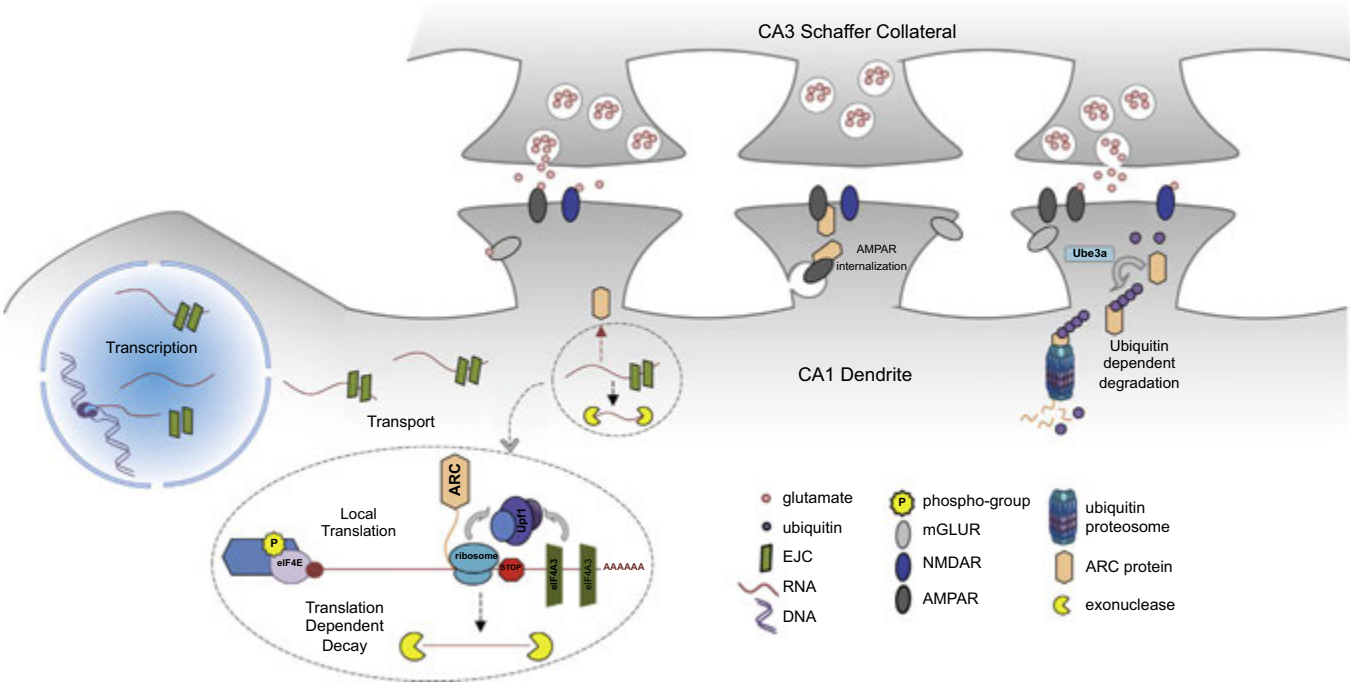

Fig. 7. Schematic of *Arc* transcription, translation and degradation pathways.

In addition to the synthesis of new proteins, degradation of existing proteins by regulated proteolysis is critical for synaptic plasticity and memory formation (for review see, Bingol & Sheng, 2011; Fioravante & Byrne, 2011; Tai & Schuman, 2008). The majority of short-lived, cytoplasmic proteins, including ARC and eIF4E (Greer et al., 2010; Murata, 2006), are degraded by the ubiquitin proteasome system (UPS). Proteins are targeted for UPS degradation by the covalent attachment of a polyubiquitin tag by one or more of the hundreds of E3 ubiquitin ligases expressed in mammals (Semple, 2003). While the overall activity of the UPS is not impaired in the aged brain, individual components of the system are altered (Cook et al., 2009; Giannini et al., 2013). Ube3a expression decreases with age in multiple species including humans (Williams, 2010), and in agreement with these findings we found that Ube3a expression is significantly decreased in the CA1 field of the hippocampus in cognitively impaired aged rats. Low Ube3a expression in this model also correlated with the severity of age-related spatial memory impairment. Deletion or loss-of-function mutations in the maternal allele of UBE3A are known to cause Angelman Syndrome (AS), which is characterized by profound cognitive impairment as well as other abnormalities (Dagli, Buiting, & Williams, 2011). The maternal allele of UBE3A is preferentially expressed in neurons as a result of tissue specific imprinting, and accordingly, mutations functionally prevent neuronal Ube3a expression (Albrecht et al., 1997). In AS mouse models, deletion of the maternal UBE3A allele causes deficits in hippocampus dependent learning and LTP (Jiang et al., 1998), blunted experience-dependent plasticity (Yashiro et al., 2009) and increased ARC protein expression (Greer et al., 2010). Interestingly, these mice also exhibit altered excitatory/inhibitory balance resulting in a net outcome that favors cortical hyper-excitability (Wallace, Burette, Weinberg, & Philpot, 2012). Deficient levels of Ube3a in CA1 of aged impaired rats may contribute to high basal ARC protein by slowing its degradation. Additionally, Ube3a has many other potential substrates that may further contribute to age-related cognitive impairment and excess neuronal excitability.

Together our results point to dysregulation of ARC protein homeostasis as an important contributor to age-related cognitive decline. In the present study we assessed several proteins known to control ARC protein abundance, however there are many other pathways and effectors that critically influence protein homeostasis that remain unexplored in the context of aging and cognition. Of particular note, additional studies are needed to determine if basal translation in aged impaired rats is broadly elevated or restricted to specific subsets of transcripts, such as dendritic mRNAs. The contributions of regulatory RNAs as well as cap-independent translation are also unknown. Exploring the role of protein homeostasis in age-related cognitive decline is an important focus for future research.

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