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Sleep loss disrupts Arc expression in dentate gyrus neurons

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ABSTRACT

Sleep loss affects many aspects of cognition, and memory consolidation processes occurring in the hippocampus seem particularly vulnerable to sleep loss. The immediate-early gene *Arc* plays an essential role in both synaptic plasticity and memory formation, and its expression is altered by sleep. Here, using a variety of techniques, we have characterized the effects of brief (3-h) periods of sleep vs. sleep deprivation (SD) on the expression of *Arc* mRNA and Arc protein in the mouse hippocampus and cortex. By comparing the relative abundance of mature *Arc* mRNA with unspliced pre-mRNA, we see evidence that during SD, increases in *Arc* across the cortex, but not hippocampus, reflect *de novo* transcription. *Arc* increases in the hippocampus during SD are not accompanied by changes in pre-mRNA levels, suggesting that increases in mRNA stability, not transcription, drives this change. Using *in situ* hybridization (together with behavioral observation to quantify sleep amounts), we find that in the dorsal hippocampus, SD minimally affects *Arc* mRNA expression, and decreases the number of dentate gyrus (DG) granule cells expressing *Arc*. This is in contrast to neighboring cortical areas, which show large increases in neuronal *Arc* expression after SD. Using immunohistochemistry, we find that Arc protein expression is also differentially affected in the cortex and DG with SD - while larger numbers of cortical neurons are Arc+, fewer DG granule cells are Arc+, relative to the same regions in sleeping mice. These data suggest that with regard to expression of plasticity-regulating genes, sleep (and SD) can have differential effects in hippocampal and cortical areas. This may provide a clue regarding the susceptibility of performance on hippocampus-dependent tasks to deficits following even brief periods of sleep loss.

1. Introduction

Over the past century, numerous studies have shown that following memory encoding, sleep promotes information storage in the brain. This has led to numerous theories of how sleep could facilitate plasticity of synapses between neurons involved in memory formation (Puentes-Mestril and Aton, 2017). Memory processes relying on neural circuits in the hippocampus (e.g., the formation of new episodic and spatial memories) seem particularly susceptible to disruption by post-encoding sleep loss (Prince and Abel, 2013).

One strategy for understanding sleep's role in brain function has been to determine how sleep and sleep loss affect gene expression in the brain. Expression of the immediate-early gene *Arc* is consistently increased in various mammalian brain structures following a period of sustained wake, relative to a similar period of *ad lib* sleep (Cirelli, Gutierrez, & Tononi, 2004; Mackiewicz et al., 2007; Vecsey et al., 2012). Arc protein function is linked to various types synaptic plasticity, with loss of *Arc* leading to deficits in long term depression (LTD) (Waung, Pfeiffer, Nosyreva, Ronesi, & Huber, 2008), late phase long term potentiation (LTP) (Messaoudi et al., 2007; Plath et al., 2006), and homeostatic plasticity (Gao et al., 2010; Shepherd et al., 2006). *Arc* mRNA and Arc protein expression are induced in specific brain circuits *in vivo* by increased neuronal activity (Miyashita et al., J Neurosci 2009) or by prior learning (Carter, Mifsud, & Reul, 2015; Czerniawski et al., 2011; Fellini and Morellini, 2013; Guzowski, Setlow, Wagner, & McGaugh, 2001). *Arc* mutants also show deficits in both hippocampus-dependent long-term memory consolidation (Plath et al., 2006) and experience-dependent plasticity in sensory cortex (McCurry et al., 2010). The level of expression of *Arc* mRNA in the hippocampus immediately following training on a hippocampus-dependent task is a predictor of subsequent memory performance (Guzowski et al., 2001).

Together the available data suggest a causal role for Arc in both synaptic plasticity and long-term memory formation. Thus a parsimonious interpretation of prior gene expression studies, showing sleep-dependent decreases in *Arc* expression (Cirelli et al., 2004; Mackiewicz et al., 2007; Vecsey et al., 2012), is that synaptic plasticity is generally decreased during sleep vs. wake. However, recent data have suggested that both functional and structural plasticity in the dorsal hippocampus

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are disrupted by sleep deprivation (Havekes et al., 2016; Vecsey et al., 2009). Other studies have shown that expression of various immediate early genes can actually be augmented (rather than reduced) in the hippocampus during post-learning sleep (Calais, Ojopi, Morya, Sameshima, & Ribeiro, 2015; Ribeiro, Goyal, Mello, & Pavlides, 1999; Ulloor and Datta, 2005). Furthermore, dorsal hippocampal network activity patterns (e.g. oscillations) associated with sleep promote both changes in functional connectivity following learning, and long-term memory formation (Ognjanovski et al., 2017; Ognjanovski, Maruyama, Lashner, Zochowski, & Aton, 2014). It is unclear whether these changes are unique to the hippocampus, the dorsal hippocampus, or perhaps to specific areas within the dorsal hippocampal circuit. To further clarify this issue, we assessed the effects of sleep and sleep loss on *Arc* mRNA expression and Arc protein levels in the hippocampus and cortex, using multiple techniques.

2. Materials and methods

2.1. Mouse handling and husbandry

All animal husbandry and surgical/experimental procedures were approved by the University of Michigan Institutional Animal Care and Use Committee (PHS Animal Welfare Assurance number D16-00072 [A3114-01]). All mice were individually housed in standard caging with beneficial environmental enrichment (nesting material and/or manipulanda) throughout all procedures. Lights were maintained on a 12 h:12 h light: dark cycle (lights on at 8 AM), and food and water were provided *ad lib*. At age 3 months, C57BL/6J mice (IMSR_JAX:000664, Jackson) were habituated to handling over 5 days, for 4 min each day. Following habituation, and beginning at lights-on (ZT0), mice either were allowed *ad lib* sleep in their home cage (Sleep) or were sleep deprived by gentle handling (SD). In the Sleep group, sleep behavior was scored based on visual observation, at 5-min intervals, throughout the 3-h *ad lib* sleep period. Criteria for sleep were immobility and a stereotyped sleep posture. Such criteria have been used (and validated against EEG-based sleep assessments) in previous studies to quantify sleep behavior in mice (Fisher et al., 2012; Pack et al., 2007). Sleep amounts across the *ad lib* sleep period are shown for individual mice in Fig. 2. Based on these scoring criteria, all Sleep mice slept > 50% of the 3-h sleep period (avg = 79%, SEM = 4%). At ZT3, mice were sacrificed by cervical dislocation under isoflurane anesthesia.

2.2. Quantitative real-time PCR (qPCR)

Whole hippocampi (and for comparison, whole cerebral cortices) from individual mice in Sleep ($n = 5$) and SD ($n = 5$) groups were dissected in PBS, flash frozen in liquid nitrogen, and stored at −80 °C. RNA purification was performed using an RNeasy Mini Kit (Qiagen) and coupled with a DNAse digestion step (Qiagen); RNA concentration and purity were quantified with spectrophotometry (Nanodrop Lite; ThermoFisher). 0.5 µg of RNA was used to synthesize cDNA using iScript cDNA Synthesis Kit (Bio-Rad) and cDNA was diluted 1:10 for mature *Arc* mRNA quantification and, due to the low expression of pre-mature Arc mRNA transcript, cDNA was diluted 1:5. qPCR reactions were measured using a CFX96 Real-Time System, in 96-well reaction plates (Bio-Rad). Three technical replicates were used for each sample. Primer specificity was confirmed using NIH Primer Blast while primer efficiency was measured by calculating primer amplification efficiency (AE) and coefficient of correlation for a standard curve (R^2) for each primer set (data and sequences shown in Table 1). All primers utilized had amplification efficiency values within 90–110%, R^2 values greater than 0.98, and standard deviations < 0.20 among 5 replicates. Expression data for *Arc* primer sets were normalized to gamma actin (*Actg1*). Expression of *Actg1* itself was not affected by SD (raw CT values: Sleep = 23.91 ± 0.15 vs. SD = 23.89 ± 0.11, *N.S.*, Student's *t*-test). *Actg1* expression levels were also normalized to the housekeeping genes *Tuba4a* (Sleep = 1.00 ± 0.035 vs. SD = 1.05 ± 0.035, *N.S.*, Student's *t*-test) and *Gapdh* (Sleep = 1.02 ± 0.093 vs. SD = 1.04 ± 0.096, *N.S.*, Student's *t*-test) and were similar between groups. For comparison with previous studies, values for SD mice were expressed as fold changes normalized to the mean values for mice in the Sleep group. Values were calculated using the delta delta Ct method.

2.3. Rnascope in situ hybridization

In situ hybridization was performed on 12-µm sections taken from fresh-frozen brains containing dorsal hippocampus, from mice in Sleep ($n = 6$) and SD ($n = 5$) groups. The RNAscope Multiplex Fluorescent Reagent Kit v2 (323100-USM, Advanced Cell Diagnosis) was used to image *Arc* expression. Prior to probe incubation, slices were pretreated with hydrogen peroxide (10 min, room temperature), Target Retrieval Reagent (5 min, 99 °C), and RNAscope protease III (30 min, 40 °C). Slices were incubated with 20 custom-synthesized *Arc* mRNA probes (316911-C3, Advanced Cell Diagnostics) targeting regions between bases 23 and 1066 within the open reading frame and hybridized to Opal 690 (PerkinElmer FP1497001KT) for visualization. Positive and negative control probes were used in parallel experiments to confirm specificity of hybridization.

2.4. Immunohistochemistry

For immunohistochemical quantification of Arc protein levels, mice in Sleep ($n = 5$) and SD ($n = 5$) groups were sacrificed and perfused with PBS followed by 4% paraformaldehyde. 40-µm brain sections were blocked with normal goat serum for 2-h and incubated overnight with a polyclonal guinea pig Arc antibody (Synaptic Systems, 156 004, 1:500) at 4 °C. The following day, sections were stained with goat anti-guinea pig IgG H&L, Alexa Fluor® 594 (Abcam, ab150188, 1:200). Stained sections were coverslipped in ProLong Gold Antifade Reagent (ThermoFisher, P36930). Fluorescence intensity was used to identify Arc protein-expressing (Arc+) cells in the dentate gyrus granule cell layer in sections containing either dorsal (−1.5 to −2.3 mm posterior to bregma) or intermediate (−2.9 to −3.2 mm posterior to bregma) hippocampus, and Arc+ neurons in posterior parietal and primary somatosensory cortical areas overlying dorsal hippocampus (1.5–3.0 mm lateral, −1.5 to −2.1 mm posterior to bregma), using the automated protocol described below.

2.5. Imaging and quantification

RNAscope probe fluorescence signals were captured using a 10× objective lens on a Leica 3D STED SP8 while immunohistochemical sections were imaged on a Leica SP5 laser scanning confocal microscope. Settings were fixed for each imaging session. Fluorescence images were analyzed using MIPAR image analysis software in their raw grayscale format (Sosa et al., 2014). Mean fluorescence intensity values (0–255) were quantified within posterior parietal and primary somatosensory cortical areas overlying the dorsal hippocampus, across granule (dentate gyrus) or pyramidal (CA1, CA3) cell layers (layer borders were delineated using a freehand tool by a scorer blind to experimental condition). In addition to layer-specific mean intensity measures, in the case of the dentate gyrus and cortex (where cell bodies were sparsely labelled with intense fluorescence), Arc-immunopositive (Arc+) cell bodies were counted by a blind scorer using an automated protocol and normalized to the area of the DG or cortical area in each section (in mm^2). Because the borders of the DG granule cell layer were difficult to distinguish in IHC sections, Arc+ cell numbers were normalized to linear distance, using a line drawn along the hilus bordering the DG. Briefly, a non-local means filter was used to reduce image noise, and an adaptive threshold was used to identify areas > 30 µm whose mean pixel intensity was 200% of its surroundings. Four images per region (two per hemisphere) were quantified for each animal.

Table 1
qPCR primers and parameters. *FC and p-values for *Actg1*, *Gapdh*, and *Tuba4a* are for raw CT measurements for each gene. Arc values are based on normalization to *Actg1*.

Gene Name	*qPCR FC (SD/Sleep)*	*qPCR p-value (Sleep vs. SD)*	*Sleep, n*	*SD, n*	*Forward Primer*	*Reverse Primer*
Actg1	1.00*	0.477*	5	5	ACTCTTCCAGCCTTCCTTC	ATCTCCTTCTGCATCCTGTC
Gapdh	0.98*	0.308*	5	5	GTGTTTCCTCGTCCCGTAGA	AATCCGTTCACACCGACCTT
Tuba4a	1.00*	0.455*	5	5	ATGCGCGAGTGCATTTCAG	CACCAATGGTCTTATCGCTGG
Arc (Hippocampus)	1.56	0.00232	5	5	CCAGATCCAGAACCACATGAA	GAGAGTGTACCCTCACTGTATTG
Arc (Cortex)	2.08	0.000580	5	5	CCAGATCCAGAACCACATGAA	GAGAGTGTACCCTCACTGTATTG
Arc pre-mRNA (Hippocampus)	1.10	0.898	5	5	GTGGGCACTGGCACTTCACG	GGTCCTCGGTGAGCTGGCTT
Arc pre-mRNA (Cortex)	1.98	0.0999	5	5	GTGGGCACTGGCACTTCACG	GGTCCTCGGTGAGCTGGCTT

3. Results

3.1. Arc mRNA expression, but not Arc transcription rates, are increased in the hippocampus during brief sleep deprivation

To investigate how sleep and sleep deprivation affect *Arc* transcription in the hippocampus, we first quantified expression of both *Arc* pre-mRNA and mature mRNA in samples obtained from whole (dorsal + ventral) hippocampus using qPCR. Animals were habituated to daily handling, and starting at lights-on the day of tissue collection they were either were allowed 3 h of *ad lib* sleep (Sleep) or were sleep deprived for 3 h by gentle handling (SD). Consistent with previous findings from the mouse hippocampus following longer (i.e., 5-h) sleep or SD intervals (Vecsey et al., 2012), qPCR results measuring the mature Arc transcript (Fig. 1A, B) indicated that 3-h SD increased *Arc* mRNA expression ~1.5-fold in the hippocampus (Sleep = 1.02 ± 0.11 vs. SD = 1.56 ± 0.05, $p < 0.01$, Student's *t*-test).

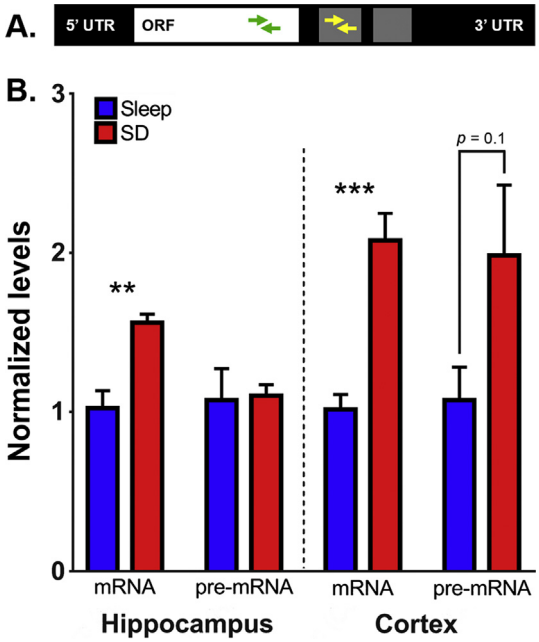

Fig. 1. Expression of mature and pre-mRNA *Arc* transcripts in hippocampus and cerebral cortex of sleep deprived animals. (A) *Arc* transcript structure and quantitative polymerase chain reaction (qPCR) primer design. To quantify *de novo Arc* transcription, *Arc* primers were designed to target either the transcript's open reading frame (green) or the first intron on its 3′ UTR (yellow). These primer sets were aimed at amplifying mature and pre-mRNA, respectively. (B) Expression of *Arc* mRNA and pre-mRNA in samples of whole hippocampus or whole cerebral cortex, normalized to expression of gamma actin (Actg1). Gene expression data in samples taken from mice after 3 h of *ad lib* sleep (Sleep) and sleep deprivation (SD) were normalized as a fold change relative to mean values from the Sleep group. Values indicate mean ± SEM; $n = 5$ mice/group; ** indicate $p < 0.01$, *** indicate $p < .001$, Student's *t*-test. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Because steady-state *Arc* mRNA levels are modulated by both transcription and degradation rates, the mechanism for *Arc* mRNA accumulation during SD is unknown. The mature *Arc* transcript is derived from a pre-mRNA containing two short introns within the 3′ UTR, which are removed during mRNA maturation (Rao et al., 2006; Saha et al., 2011). The mature mRNA has a half-life of approximately 45 min *in vivo* (Rao et al., 2006), and is thought to be degraded rapidly after translation, via translation-dependent decay (Farris, Lewandowski, Cox, & Steward, 2014; Ninomiya, Ohno, & Kataoka, 2016). To determine whether observed increases in *Arc* mRNA expression with SD were due to increased *de novo* synthesis or mature mRNA stabilization, we designed primers which spanned the first intron of the *Arc* pre-mRNA transcript. Previous *in vitro* studies using similar primers have demonstrated that *Arc* pre-mRNA expression increases 5–10 min prior to mature *Arc* mRNA (Saha et al., 2011). Using these primers (Fig. 1A) we found no significant effect of SD on hippocampal *Arc* pre-mRNA levels (Fig. 1B), which were nearly identical in Sleep and SD mice (Sleep = 1.07 ± 0.20 vs. SD = 1.10 ± 0.07, *N.S.*, Student's *t*-test). This suggests that increased expression of mature *Arc* mRNA after SD may not result from increased *de novo Arc* transcription during the wake state, as previously assumed. Rather, increased mature mRNA levels could be driven by reduced translation-dependent mRNA decay during SD.

For comparison with changes seen in the hippocampus, we also used qPCR to measure *Arc* pre-mRNA and mature mRNA in cerebral cortical samples taken from the same Sleep and SD mice (Fig. 1B). Consistent with the findings of others (Cirelli et al., 2004; Mackiewicz et al., 2007; Vecsey et al., 2012), we found that mature *Arc* mRNA was increased approximately twofold in the cortex following SD (Sleep = 1.02 ± 0.09 vs. SD = 2.08 ± 0.17, $p < 0.001$, Student's *t*-test). In contrast to pre-mRNA levels in the hippocampus, which were identical in mice from SD and Sleep groups, pre-mRNA levels in cortex measured with both primer sets were elevated following SD (Sleep = 1.07 ± 0.21 vs. SD = 1.98 ± 0.44). While this change did not reach statistical significance ($p = 0.1$), it was similar in magnitude (i.e., twofold) to the increase in mature mRNA seen in cortex with SD (Fig. 1B).

3.2. Sleep selectively increases Arc mRNA expression among neurons in the dentate gyrus

To clarify where in the hippocampal circuit *Arc* mRNA expression is regulated, we used RNAscope fluorescence *in situ* hybridization (Wang et al., 2012) to visualize the mature *Arc* transcript. To do this, we utilized predesigned Arc RNAScope probes targeting the bases 23–1066 in the Arc ORF. To ensure their specificity, positive control probes targeting the housekeeping gene *Hprt1* and negative control probes targeting *DapB* (a gene expressed in *Bacillus subtilis*) were run alongside the *Arc* probes (Fig. 3). Once the specificity of the probes were confirmed, multiple hippocampal subregions (dentate gyrus [DG], CA3, and CA1) were imaged from brain sections containing the dorsal hippocampus which were taken from mice in Sleep ($n = 6$) and SD ($n = 5$) groups. Across each area, mean fluorescence signal intensity was first

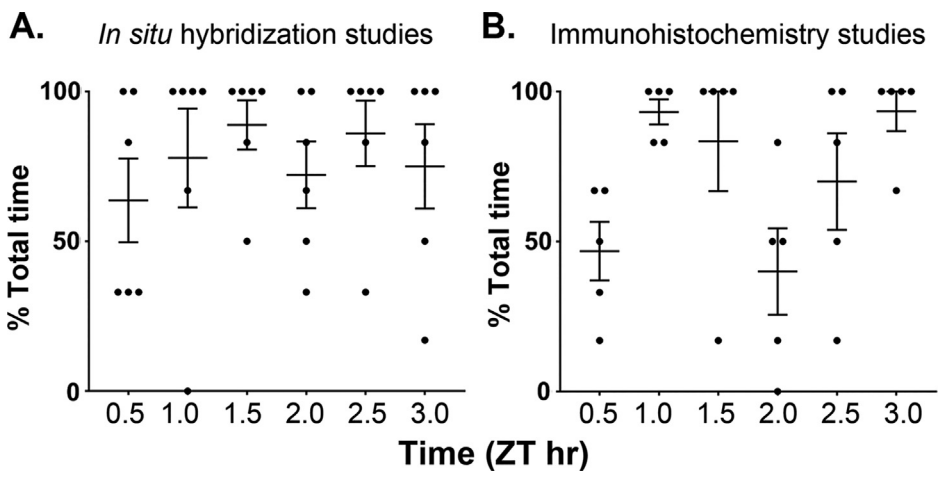

Fig. 2. Total sleep time in mice allowed *ad lib* sleep from ZT0-3. Amount of time during which Sleep mice were observed to be inactive and in stereotyped sleep posture across the 3-h *ad lib* sleep period. Values are expressed as a percentage of total time, in 30-min intervals. Data are shown for mice used for *in situ* hybridization studies in (A) and for mice used for immunohistochemistry studies in (B).

quantified in pyramidal (or granule) cell body layers, and in adjacent molecular layers (i.e., in pyramidal/granule cell dendritic fields). As shown in Fig. 4, there were no significant changes in *Arc* expression with SD in either pyramidal cell or molecular layers, in either CA1, CA3, or DG. For comparison with hippocampal expression values, we also measured *Arc* mRNA expression in cortical areas (i.e., posterior parietal and primary somatosensory cortex) overlying dorsal hippocampus. As shown in Fig. 4B, mean signal intensity (measured across all cortical layers) was slightly, but not significantly, higher following SD.

In DG and in cortex, *Arc*+ neuronal labeling was sparse, allowing comparisons of *Arc*+ neuronal density in these regions between Sleep and SD. As shown in Fig. 5A–B, we observed twice as many *Arc*+ neurons in the DG granule cell layer of mice from the Sleep group than were seen in the DG of mice from the SD group (87.44 ± 3.70 vs. 39.40 ± 7.59 cells/mm^2, $p < 0.001$, Student's *t*-test). No differences in the background mean fluorescence intensity (measured within the granule cell layer, with fluorescence intensity values of Arc+ cell bodies subtracted) were observed in DG between mice in Sleep and SD groups (data not shown, Sleep = 43.39 ± 5.32 vs. SD = 45.34 ± 4.69, *N.S.*, Student's *t*-test). This suggests that in contrast to areas CA1 and CA3 (where *Arc* mRNA expression levels are largely unchanged after SD), expression of *Arc* among DG granule cells is significantly greater following a period of *ad lib* sleep. However, DG *Arc* expression levels for individual animals were not correlated with sleep amounts across the 3-h *ad lib* sleep period prior to sacrifice (*N.S.*, Pearson correlation, Fig. 5C).

In contrast to what was seen in DG (and consistent with qPCR data from whole cortical RNA samples), *Arc*+ neuronal labeling in the cortex indicated a ~3-fold increase in *Arc* + neuronal density after SD (Fig. 5D–E; Sleep = 43.17 ± 10.76 cells/mm^2 vs. SD = 149.9 ± 19.14 cells/mm^2, $p < 0.001$, Student's *t*-test). Among freely-sleeping mice, the number of *Arc* + neurons in the cortex of individual mice was predicted by the amount of time spent awake vs. asleep over the final fifteen minutes prior to sacrifice (Fig. 5F; Pearson $R = 0.94$, Bonferroni corrected p value < 0.05).

3.3. Sleep increases Arc protein levels in the dorsal and intermediate dentate gyrus

To determine whether brain region-specific changes in *Arc* mRNA expression were mirrored by changes Arc protein levels after SD, we used immunohistochemistry to measure differences in Arc translation. As observed in the *in situ* hybridization experiments, no significant changes in the mean fluorescence intensity were recorded following SD

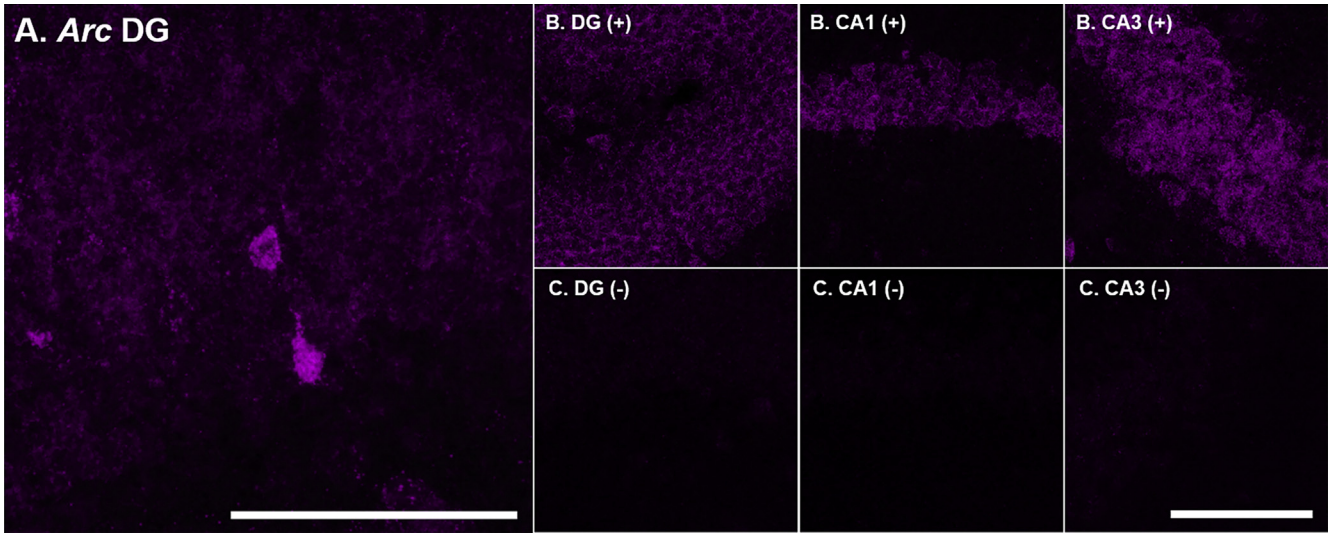

Fig. 3. Validation of *Arc* mRNA probes for RNAscope fluorescence *in situ* hybridization. (*A*) RNAscope *in situ* hybridization of mouse dentate gyrus (DG) section. Violet color represents *Arc* mRNA probe hybridization. (B) Positive control probes for *in situ* hybridization, targeting mRNA for the ubiquitously-expressed housekeeping gene *Hprt1*; images show representative signal in DG, CA1, and CA3. (C) Negative control probes targeting mRNA for *DapB*, a gene expressed in *Bacillus subtilis*, shown in the same regions. Scale bars indicate 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

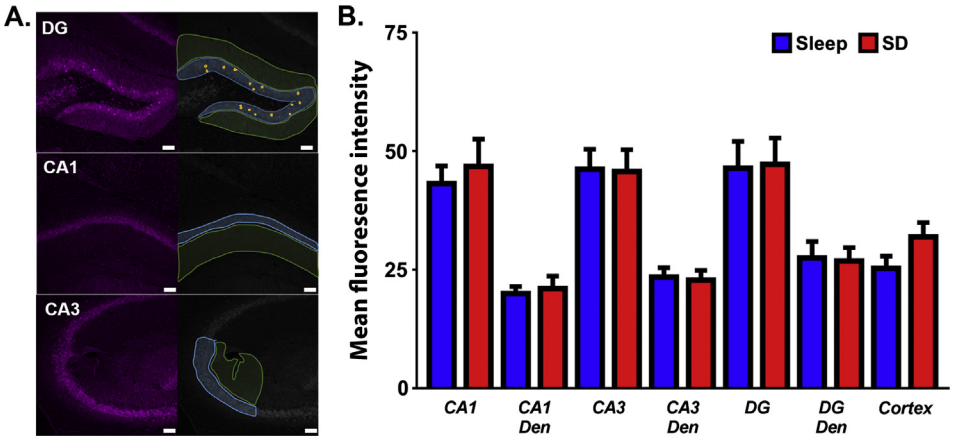

Fig. 4. Mean fluorescence intensity values for *in situ* hybridization data for *Arc* mRNA in dorsal hippocampus and cortex. (A) Strategy for measuring mean fluorescence intensity in pyramidal cell and dendritic (Den) layers in dorsal hippocampal subregions. Selection of granule cell layer of the dentate gyrus and pyramidal cell layer of CA1/CA3 are shown in blue. Estimated dendritic regions adjacent to cell layers are shown in green. For *Arc* cellular quantification in the DG, an automated protocol (see Methods) detected *Arc*+ cells in the DG (yellow) and counted the number of *Arc*+ cells/mm^2 within the granule cell layer (blue). (B) Mean fluorescence values did not significantly differ between Sleep (n = 6 mice) and SD (n = 5 mice) conditions in any area. Scale bars indicate 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

in hippocampal subregions CA1 and CA3 (Fig. 6). In contrast, as shown in Fig. 7A–B, in both dorsal (Sleep = 16.46 ± 1.84 cells/mm vs. SD = 10.96 ± 0.80 cells/mm), $p < 0.05$, Student's t-test) and intermediate (Sleep = 10.48 ± 0.69 cells/mm vs. SD = 3.22 ± 1.06 cells/mm, $p < 0.001$, Student's t-test) DG, the number of granule cells expressing Arc protein was decreased in SD mice. Among mice in the Sleep group, in both dorsal and intermediate DG, the number of Arc+ cells tended to be highest in animals that had slept the most over the last 1.25 h prior to sacrifice (Pearson R = 0.86 and 0.93, respectively, Bonferroni-corrected p value *N.S.*) (Fig. 7C). This suggests that appropriately-timed sleep may promote the translation of Arc protein among DG neurons.

For comparison, we also quantified expression of Arc protein in primary somatosensory and posterior parietal cortex. As was true for *Arc* mRNA, following SD, mice showed a ~5-fold increase in the number of Arc+ cells across all layers of the cortex (Sleep = 25.18 ± 17.49 cells/mm^2 vs. SD = 122.90 ± 27.29 cells/mm^2, $p < 0.05$, Student's t-test). Similar to results observed in the cortex, among sleeping mice, Arc protein levels tended to be reduced in animals sleeping more over the last hour prior to sacrifice (Pearson $R = -0.84$, Bonferroni-corrected p value *N.S.*).

4. Discussion

Here, we show that sleep- and SD-associated *Arc* mRNA and protein expression patterns vary between the hippocampus and cortex. We find that expression levels measured in samples of whole hippocampus (or cortex) increase in a manner consistent with previous reports (Cirelli et al., 2004; Mackiewicz et al., 2007; Thompson et al., 2010; Vecsey et al., 2012). By comparing the relative levels of pre-mRNA and mature mRNA in Sleep and SD conditions, we find new (immature) *Arc* transcripts in the hippocampus are unchanged across SD, while in the cortex, increases in pre-mRNA parallel increases in mature *Arc* mRNA. This suggests that previously-reported increases in *Arc* mRNA in cortex following SD (Cirelli et al., 2004; Mackiewicz et al., 2007) reflect *de novo* transcription.

Previous work has shown that in the hippocampus, stimulus-induced expression of mature *Arc* mRNA lags expression of its pre-mRNA by only 5–10 min (Saha et al., 2011). Our surprising finding of an increase in mature *Arc* in the hippocampus *without* a corresponding increase in pre-mRNA suggests that a non-transcriptional mechanism must increase *Arc* levels across SD. One mechanism which could plausibly affect the ratio of pre-mRNA to mature RNA is an altered rate of

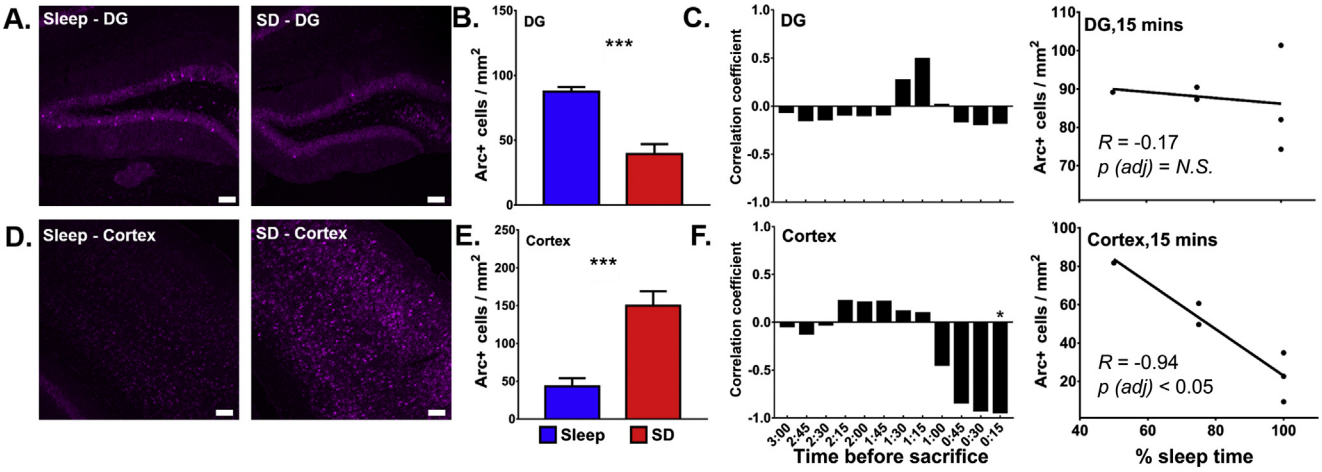

Fig. 5. Sleep deprivation simultaneously decreases *Arc*+ cells in the DG, and increases *Arc*+ cells in the cortex. (A) Representative images showing *Arc*+ cells in the DG following 3 h of *ad lib* Sleep (n = 6 mice) or SD (n = 5 mice). Scale bars indicate 100 μm. (B) The number of *Arc*+ cells/mm^2 was reduced in the DG of mice following SD mice relative to mice allowed *ad lib* Sleep. Values indicate means ± SEM for each condition; *** indicates $p < 0.001$, Student's t-test. (C) Pearson correlation coefficients for *Arc*+ cells/mm^2 in DG vs. sleep time integrated over various intervals prior to sacrifice, based on sleep amounts from individual mice in the Sleep condition. (D) Representative images from Sleep and SD mice, showing *Arc*+ cells in primary somatosensory cortex overlying dorsal hippocampus. Scale bars indicate 100 μm. (E) The number of *Arc*+ cells/mm^2 in the cortex was increased after SD. *** indicates $p < 0.001$, Student's t-test. (F) Pearson correlation coefficients for cortical *Arc*+ cells/mm^2 vs. total sleep time, integrated over various intervals prior to sacrifice. Negative relationships between sleep time and *Arc*+ cell numbers were present over the final 45-min of the experiment (* indicates $p < 0.05$ after Bonferroni correction).

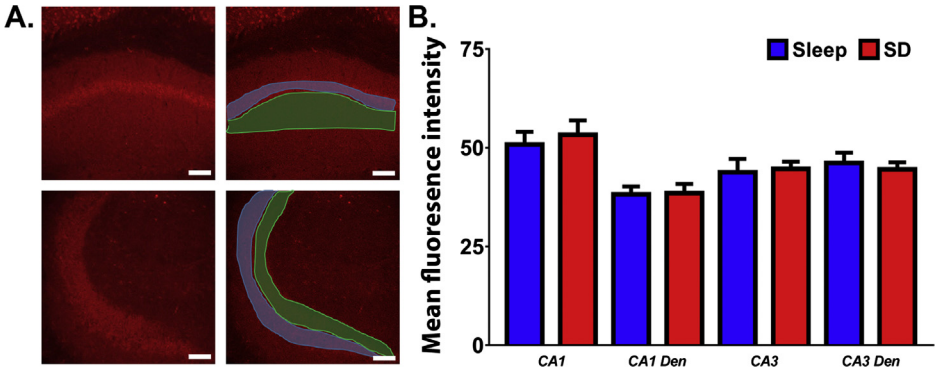

Fig. 6. Sleep deprivation does not significantly alter Arc protein levels in hippocampal areas CA3 or CA1. (A) Strategy for measuring mean fluorescence intensity in pyramidal cell and dendritic (Den) layers in dorsal hippocampal subregions. Selection of the pyramidal cell layer in CA1/CA3 are shown in blue. Estimated dendritic regions adjacent to cell layers are shown in green. (B) Mean fluorescence values did not significantly differ between Sleep (n = 5 mice) and SD (n = 5 mice) conditions in any area. Scale bars indicate 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

pre-mRNA splicing. Here, we would expect an increase in the splicing rate in the hippocampus with SD. While splicing rates can be altered *in vitro* (for example, through phosphorylation of the C-terminal of RNA polymerase II (Millhouse and Manley, 2005), the rate of splicing *in vivo* is tightly coupled with polymerase recruitment, and thus the rate of transcription (Saldi, Cortazar, Sheridan, & Bentley, 2016). Furthermore, expression of genes required for RNA splicing are reportedly down-regulated in the hippocampus across a period of SD (Vecsey et al., 2012), making this an unlikely explanation for our results. In contrast, selective increases in levels of mature mRNA could be explained by increased mRNA stability. There is abundant evidence that following translation, mature *Arc* transcripts undergo rapid degradation; thus the half-life of new hippocampal *Arc* transcripts is ~45 min (Rao et al., 2006). One possibility is that during SD (and specifically in the hippocampus), the activity- and translation-dependent mechanisms which mediate Arc mRNA degradation (Farris et al., 2014; Ninomiya et al., 2016) are suppressed. Prior data have suggested that translation rates are decreased throughout the brain during spontaneous wake relative to slow wave sleep (Ramm and Smith, 1990), and that SD suppresses protein synthesis in the hippocampus (Tudor et al., 2016; Vecsey et al., 2012). Further, sleep promotes, and SD reduces, the expression of genes involved in protein synthesis (Mackiewicz et al., 2007). Thus a parsimonious explanation of our current findings is that in the hippocampus, SD does not increase the rate of *Arc* transcription, but slows its translation and subsequent degradation. Our immunohistochemical results are consistent with this interpretation; expression of Arc protein in the DG is reduced after just 3 h of SD.

Arc is an immediate early gene which is rapidly transcribed in the hippocampus response to increasing neural activity (Rao et al., 2006) and learning (Guzowski et al., 2001). SD in the hours following learning is known to disrupt memory consolidation for tasks that are selectively dependent on the dorsal hippocampus (Graves, Heller, Pack, & Abel, 2003; Vecsey et al., 2009). Thus we assessed how SD affected *Arc* mRNA expression among neurons in specific subregions of the dorsal hippocampus and in overlying neocortex, using fluorescence *in situ* hybridization. Surprisingly, we found that no significant changes in overall expression levels with SD in any of these regions. Mean intensity values were nearly identical in Sleep and SD conditions in CA1, CA3, and DG, and while expression tended to be higher in the cortex following SD, intensity differences between Sleep and SD were not statistically significant. We attribute the lack of statistical significance for changes in overall *in situ* fluorescence (when changes were detected using qPCR) to two plausible issues. First, the quantified areas were necessarily subsampled for *in situ* hybridization, while qPCR quantified expression in whole brain structures. Second, the differential outcomes may be the result of differences in the sensitivity of detection using the two techniques. In contrast, by counting the density of *Arc*+ neurons in DG and cortex, we found substantial differences in expression between Sleep and SD conditions. In cortex, these changes mirrored changes in cortical *Arc* mRNA expression levels measured using qPCR - with higher numbers of neurons labeled with *Arc* probes after SD. Arc protein levels follows the same pattern in the cortex, with significantly higher expression following SD, and a negative relationship between sleep time and the number of Arc-expressing neurons. In stark contrast, significantly fewer DG granule cells were *Arc*+ in the SD condition. Together these data suggest that sleep, but not extended wake, supports *Arc* expression in the DG; further, this relationship between sleep and *Arc* expression may be unique to DG granule cells. Our immunohistochemical quantification of Arc protein in the DG supports this idea - we find that Arc protein-expressing granule cells are also more numerous following a 3-h interval of *ad lib* sleep that following SD, and that expression levels showed a strong tendency to be higher in animals that had spent more time sleeping.

Ours is not the first study to link DG *Arc* expression to sleep. Recently, Renouard et al. demonstrated that following a prolonged (multi-day) period of REM sleep deprivation, *Arc* expression is reduced in the hippocampus (and increased in the cortex); during subsequent recovery sleep (which contains relatively high amounts of REM) *Arc* levels increase and decrease, respectively, in hippocampus and cortex. The same study demonstrated immunohistochemically that the number of Arc+ neurons in DG decreased with REM sleep deprivation, and increased with recovery sleep (Renouard et al., 2015). The authors attributed these changes to differing amounts of REM in the various experimental conditions. However, another possibility is that these changes were related to differences in the amount of non-REM (NREM) sleep and wake, which were significantly decreased and increased, respectively, as a result of REM sleep deprivation (Renouard et al., 2015). While mice in our present study were not instrumented for polysomnographic quantification of REM and NREM sleep, we would expect (based on prior studies of *ad lib* sleep in instrumented C57Bl6/J mice) that REM constitutes roughly 5–10% of total recording time at ZT0-3, while NREM constitutes 50–70% of the same time period (Huber, Deboer, & Tobler, 2000; Koehl, Battle, & Meerlo, 2006; Meerlo, Easton, Bergmann, & Turek, 2001; Wimmer, Rising, Galante, Wyner, & Abel, 2013). The limited REM sleep time expected for mice in our current study (which we estimate would amount to 9–18 min, total, prior to sacrifice) suggest that either REM sleep can induce expression of Arc in the DG very efficiently and quickly, or that NREM sleep may also be important for Arc expression. While future studies will be required to address this issue, it is clear from our present findings that even brief periods of sleep loss disrupt DG Arc expression.

How might increased DG expression of Arc during sleep impact hippocampal function? In the absence of Arc expression, various forms of synaptic plasticity are disrupted, including homeostatic plasticity, LTD, and LTP (Gao et al., 2010; Messaoudi et al., 2007; Plath et al., 2006; Shepherd et al., 2006; Waung et al., 2008). In the DG, Arc plays a role in synaptic structure as well as function. Disruption of Arc in the DG leads to reduced phosphorylation of the actin depolymerization factor cofilin, and reduced synaptic filamentous actin (F-actin) (Messaoudi et al., 2007). Since SD appears to disrupt *Arc* transcription and translation among granule cells in a similar manner, one might expect reduced synaptic spine density in DG after SD. Indeed, in this

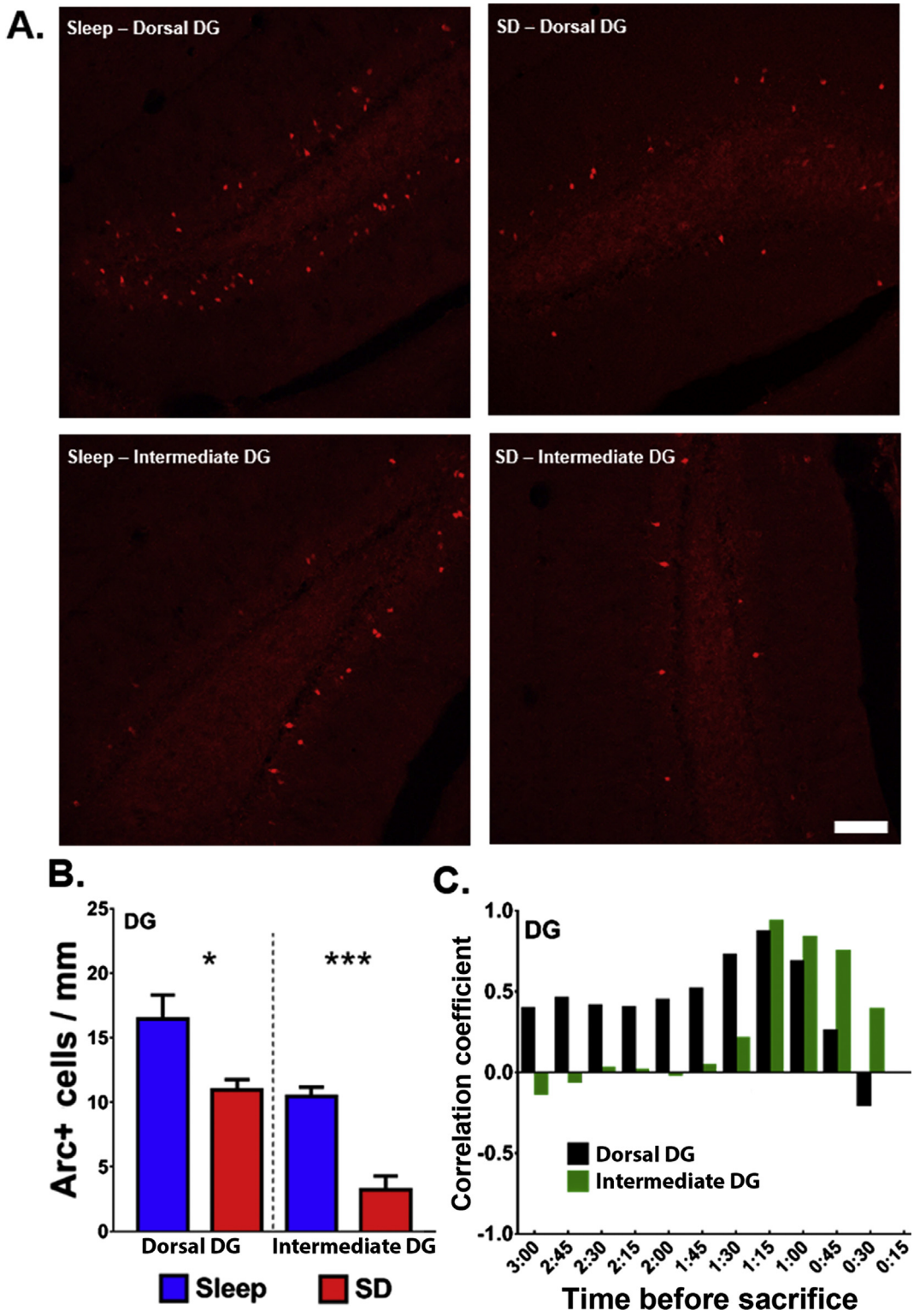

Fig. 7. Sleep deprivation simultaneously decreases Arc protein levels among DG cells, and increases Arc expression among cortical cells. (A) Representative images of immunohistochemical staining for Arc in dorsal and intermediate DG following 3 h of *ad lib* Sleep ($n = 5$ mice) or SD ($n = 5$ mice). (B) Arc+ cells/mm were decreased in both dorsal and intermediate DG. Data indicate mean ± SEM for each condition; * indicates $p < 0.05$, *** indicates $p < 0.001$, Student's *t*-test. *C.* Pearson correlation coefficients for cortical Arc+ cells/mm in dorsal (black bars) and intermediate (green bars) DG vs. sleep time integrated over various intervals prior to sacrifice. (D) Representative images from Sleep and SD mice, showing Arc+ cells in primary somatosensory cortex overlying dorsal hippocampus. Scale bar indicates 100 μm. *E.* The number of Arc+ cells/mm^2 in the cortex was increased after SD. * indicates $p < 0.05$, Student's *t*-test. (F) Pearson correlation coefficients for cortical Arc+ cells/mm^2 vs. sleep time integrated over various intervals prior to sacrifice. Negative relationships between total sleep time and *Arc+* cell numbers were present over the final hour of the experiment. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

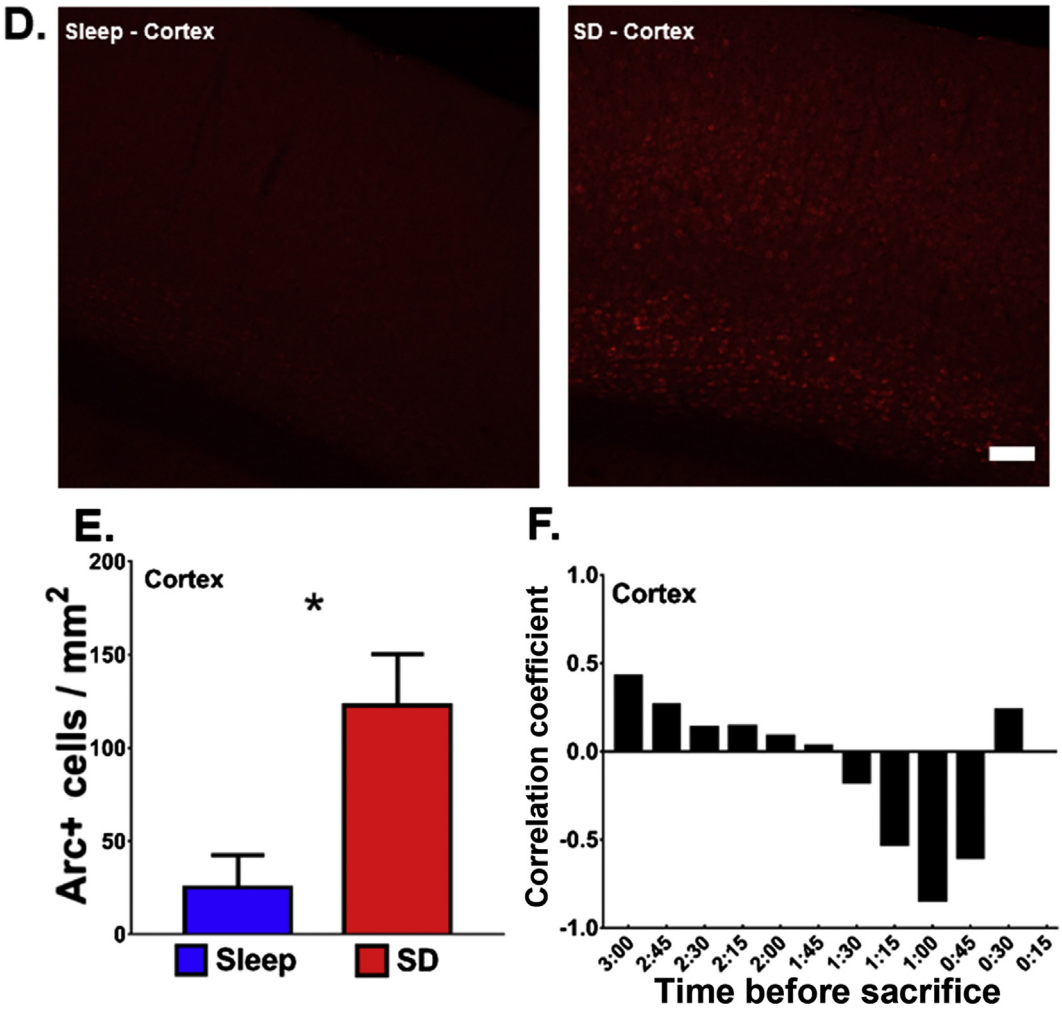

Fig. 7. (*continued*)

same issue, Raven et al. show that spine density in DG granule cells is reduced after 5 h of SD (Raven et al., 2018). Prior work has shown that SD also reduces cofilin phosphorylation, leading to reduced spine numbers (Havekes et al., 2016). Taken together, the available data suggest that SD disrupts Arc-dependent regulation of the actin cytoskeleton in the DG, and that this leads to reduced dendritic spine numbers among granule cells. Because DG granule cells play a critical role in the recall of spatial, episodic, and contextual memories (Bernier et al., 2017; Liu et al., 2012; Morris, Curtis, Chruchwell, Maasberg, & Kesner, 2013; Niewoehner et al., 2007; Yokoyama and Matsuo, 2016), it is plausible that deficits in hippocampally-mediated cognitive functions after SD are mediated in part by effects on Arc expression in DG.

A final unresolved question is why Arc expression is reduced in DG, while simultaneously being increased in cortex, following brief SD. Previous studies, using longer periods of SD (up to 24 h), have shown differential effects on dendritic spines in other areas of hippocampus (e.g., CA1) vs. cortex (Acosta-Pena et al., 2015; Havekes et al., 2016). We have proposed previously that sleep may have differing effects on intracellular pathways required for synaptic plasticity, either down-regulating or upregulating their activity, depending on prior experience during wake (Puentes-Mestril and Aton, 2017). This idea is based on experimental findings from studies carried out using animal models over the past two decades. For example, following a learning experience, immediate early genes *Egr1, Fos, and Arc* (Calais et al., 2015; Ribeiro et al., 1999) and Arc protein (Ulloor and Datta, 2005) expression are increased in the hippocampus during subsequent sleep. Data from our own lab suggest that both sleep-associated network activity patterns (Aton et al., 2013; Aton, Suresh, Broussard, & Frank, 2014; Durkin and Aton, 2016; Durkin et al., 2017; Ognjanovski et al., 2014, 2017) and sleep-associated activation of cellular signaling pathways involved in synaptic plasticity (Aton et al., 2009) vary as a function of prior learning experience.

Multiple lines of evidence suggest that DG synaptic plasticity, in particular, may be augmented preferentially during sleep. It has long been known that following spatial task performance, DG place cell reactivation occurs selectively during NREM sleep (Shen, Kudrimoti, McNaughton, & Barnes, 1998). Since the DG appears to play a continuous role in encoding and storing spatial, temporal, and contextual aspects of the animal's environment (Kesner, 2018), it seems likely that mechanisms underlying synaptic- and systems-level memory consolidation (including those mediated by Arc) would be active in this structure during sleep, even under "baseline" conditions. DG granule cells integrate inputs from cortical, hippocampal, and septal structures (Kesner, 2018), all of which show sleep-associated changes in activity (Puentes-Mestril and Aton, 2017). Recent studies support the idea that cortical input to the DG is altered, and possibly augmented, during sleep. For example, spikes of highly synchronous DG activity occur frequently during NREM sleep, and that these spikes are temporally associated with inter-regional cortical up-states (Headley, Kanta, & Pare, 2017). Theta (7–12 Hz) and slow oscillatory (~1 Hz) activity patterns (associated with REM and NREM, respectively) differentially modulate input to the DG via the lateral and medial perforant pathway (Schall and Dickson, 2010). Finally, during NREM sleep, DG evoked firing rate responses to input are higher than during wake (Winson and

Abzug, 1978). Based on our present data and these prior findings, we hypothesize that the unique network connectivity of the DG leads to activity-driven plastic changes – mediated, at least in part, by Arc - in this structure during sleep.

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Conflict of interest

The authors declare no conflict of interest.

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