Loss of *Arc* attenuates the behavioral and molecular responses for sleep homeostasis in mice

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The activity-regulated cytoskeleton-associated protein (*Arc*) gene is a neural immediate early gene that is involved in synaptic downscaling and is robustly induced by prolonged wakefulness in rodent brains. Converging evidence has led to the hypothesis that wakefulness potentiates, and sleep reduces, synaptic strengthening. This suggests a potential role for *Arc* in these and other sleep-related processes. However, the role of *Arc* in sleep remains unknown. Here, we demonstrated that *Arc* is important for the induction of multiple behavioral and molecular responses associated with sleep homeostasis. *Arc* knockout (KO) mice displayed increased time spent in rapid eye movement (REM) sleep under baseline conditions and marked attenuation of sleep rebound to both 4 h of total sleep deprivation (SD) and selective REM deprivation. At the molecular level, the following homeostatic sleep responses to 4-h SD were all blunted in *Arc* KO mice: increase of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor GluA1 and its phosphorylation in synaptoneurosomes; induction of a subset of SD-response genes; and suppression of the *GluA1* messenger RNA in the cortex. In wild-type brains, SD increased Arc protein expression in multiple subcellular locations, including the nucleus, cytoplasm, and synapse, which is reversed in part by recovery sleep. *Arc* is critical for these behavioral and multiple molecular responses to SD, thus providing a multifunctional role for *Arc* in the maintenance of sleep homeostasis, which may be attributed by the sleep/wake-associated changes in subcellular location of Arc.

sleep homeostasis | nuclear translocation | activity-regulated cytoskeleton-associated protein | GluA1 | immediate early gene

Introduction

Sleep is homeostatically regulated. A hypothetical substrate of sleep need accumulates in the central nervous system during waking and resolves during sleep (1). Although the molecules or genes involved in sleep need remain unclear, electroencephalography (EEG) delta power (0.5–4 Hz) in non–rapid eye movement (NREM) sleep is well accepted as the most reliable marker of sleep need (1). In response to sleep loss, homeostatic sleep regulation induces sleep rebound and elevated delta power in NREM sleep to compensate for the loss (1). A broad range of molecular events associated with energy metabolism, synaptic plasticity, and macromolecule biosynthesis also occur in response to sleep loss to maintain sleep homeostasis (2). These events are the three major theoretical reasons for sleep, which are supported by the up-regulation of key genes or proteins associated with those events in the brain (2). Currently, based on several lines of evidence, the theoretical model of the relationship between sleep and synaptic plasticity has been developed as the synaptic homeostasis hypothesis (SHY, ref. 3). The SHY posits that synaptic down-regulation is a critical functional characteristic of sleep: the experience during waking increases synaptic strength, and sleep eliminates its excess (3). In sleep-deprived rodent brains, increases in spine number and volume (3, 4), in axon–spine interface (4), in total and phosphorylated GluA1 α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor levels in synaptoneurosomes (5), in synaptic strength (3, 6), and in phosphorylation levels of a subset of synaptic proteins (7) have been reported. In addition to the correlation between these genes/proteins and sleep need, recent omics studies reported an increase in the expression of several immediate early genes in rodent brains exposed to prolonged waking conditions (8–10). However, the direct function of these genes that underlie sleep regulation remains to be elucidated.

Arc, one of such neuronal immediate early genes, is dramatically induced in sleep-deprived brains; it is up-regulated during both forced and spontaneous waking and down-regulated during sleep in the brain (8, 11–13), especially in the cortex and hippocampus (14), where EEG delta and theta oscillations are most robustly measured, respectively. EEG delta power is highest during NREM sleep, while EEG theta power is greatest during REM sleep. These previous reports suggest a potential association between the *Arc* gene and sleep homeostasis; however, *Arc* has been considered merely as a sleep marker, because of the poor evidence regarding its function in sleep (11, 13), such as *Arc*'s involvement in sleep architecture, sleep-related gene expression, sleep-related synaptic scaling, or sleep homeostasis.

In contrast, many studies have implicated *Arc* as a central player in the synaptic plasticity involved in learning and memory processes (15, 16). The expression of the *Arc* messenger RNA (mRNA) is tightly regulated by neuronal activity (17, 18). The mRNA is transported to dendrites and translated to the encoded

Significance

***Arc* is a neural immediate early gene involved in synaptic downscaling and is robustly induced by prolonged wakefulness in rodent brains. However, it remains unclear if and how *Arc* is involved in sleep regulation. Here we find that *Arc* is important for inducing multiple homeostatic responses induced by sleep deprivation at behavioral and molecular levels: (1) rebound of sleep time; (2) expression of a subset of sleep deprivation-induced genes; and (3) synaptic glutamate receptor expression. In sleep-deprived wild-type brains, Arc protein levels are significantly increased in the nucleus, cytoplasm, and synapse, suggesting multiple roles for Arc depending on its subcellular location. These findings provide the functional evidence for the role of *Arc* in homeostatic sleep regulation.**

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protein locally (18, 19). At the synapse, Arc is involved in long-term potentiation (LTP) consolidation (20), synaptic number and morphology (21), and late-phase LTP (22), which mediates synaptic scaling. Thus, *Arc* seems to be deeply involved in synaptic strengthening. However, studies have demonstrated that Arc exists in the cytoplasm of excitatory neurons in silent synapses (23) and down-regulates synaptic strength by reducing spine number and increasing AMPA receptor trafficking (16, 24). In addition, neural activities also regulate Arc nuclear shuttling (25). In nuclei, Arc establishes a complex with promyelocytic leukemia protein nuclear bodies (PML-NBs) as a polymer of a transcriptional cofactor (26, 27); in turn, this complex down-regulates the *GluA1* mRNA presumably via an epigenetic mechanism (25, 28). Consequently, nuclear Arc reduces *GluA1* mRNA translation (25). Thus, currently, it is widely accepted that synaptic down-regulation is the most important function of *Arc*. However, these opposing findings of Arc suggest the possibility that Arc may contribute to sleep/wake-associated synaptic homeostasis bidirectionally, or in either direction of synaptic upscaling or weakening, as proposed by the SHY.

In this study, to investigate the mechanisms underlying the role of *Arc* in sleep homeostasis, we first evaluated the sleep phenotype in constitutive *Arc* knockout (KO) mice (29) under baseline conditions, as well as in response to continuous 4-h total sleep deprivation (SD) and selective REM deprivation. Subsequently, we assessed *Arc* involvement in the associated molecular events that occur in response to SD: SD-induced sleep-related gene expression, synaptic GluA1 expression, and Arc nuclear distribution within the context of the homeostatic sleep response. We report that, in the absence of *Arc*, all of the sleep homeostatic responses described above were disrupted at both the behavioral and molecular levels.

Results

Sleep Phenotype and Homeostasis in *Arc* KO Mice. At baseline, *Arc* KO mice showed a normal diurnal sleep pattern over 24 h (Fig. 1 *A–C*, *P*, and *T*), yet exhibited an increased total time in REM sleep in the light phase due to increased REM sleep episode numbers compared to wild-type (WT) mice (Fig. 1*C* and *SI Appendix*, Fig. S1 *B*, *Left*). In contrast, there were no differences between the genotypes in the parameters associated with baseline

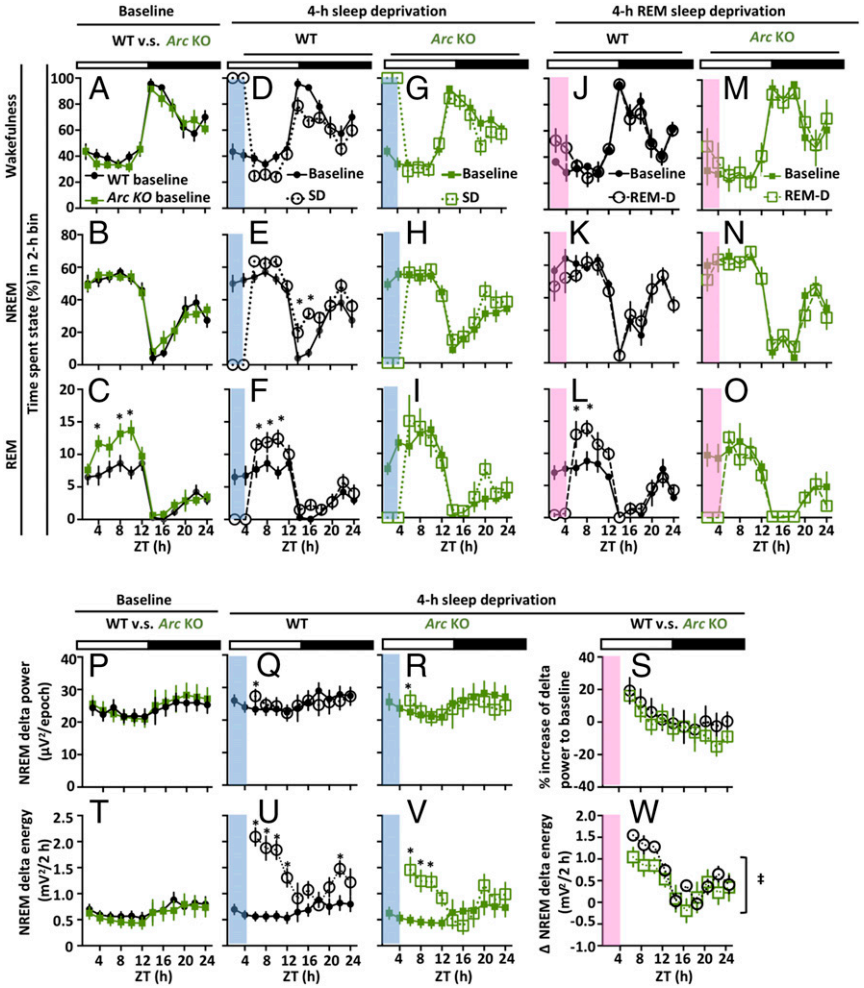

Fig. 1. Sleep phenotype and behavioral homeostatic sleep response in *Arc* KO mice. (*A–C*) Percentages of time spent in wakefulness (*A*), NREM sleep (*B*), and REM sleep (*C*) across 24 h baseline in WT (black, $n = 8$) and *Arc* KO (green, $n = 7$) mice. (*D–I*) Percentage of time spent in states at baseline (solid) and after SD (dashed) in response to 4 h of SD (blue shading) for WT (open circle, $n = 8$) and *Arc* KO (open square, $n = 7$) mice. (*J–O*) Baseline and response to 4-h selective REM deprivation (REM-D, pink shading) in states in WT ($n = 4$) and *Arc* KO ($n = 4$) mice. (*P–R* and *T–V*) Delta power (*Top*) and NREM delta energy (*Bottom*) of baseline (*P* and *T*) and 4-h SD (*Q*, *R*, *U*, and *V*). (*S* and *W*) Direct comparisons between genotypes of changes in delta power (*S*) and NREM delta energy (*W*) following 4-h SD relative to control condition. (*W*) During recovery sleep, changes in the delta energy in *Arc* KO mice were significantly lower than those detected in WT mice (F [1, 116] = 5.60, $P = 0.020$, two-way ANOVA). All averaged values were plotted in 2-h intervals. $^*P < 0.05$ compared with time-matched baseline by Sidak's multiple-comparisons test following two-way ANOVA; $^‡P < 0.05$ compared between genotypes by two-way ANOVA.

NREM sleep: total time spent in NREM sleep (Fig. 1*B*), NREM sleep episode frequency (see *SI Appendix*, Fig. S1*A*), and 24-h delta power distribution (Fig. 1*P*). Next, to examine the homeostatic sleep response, continuous SD was employed from ZT0–4, and recovery sleep responses were observed during the recovery period, from ZT4–24. In *Arc* KO mice, SD induced no appreciable rebound in NREM or REM sleep amount (Fig. 1 *H* and *I*). Interestingly, however, they exhibited an apparently normal increase in NREM delta power following 4-h SD (Fig. 1*R*). Overall, a lower level of NREM delta energy was detected in *Arc* KO mice compared to WT (Fig. 1*W*). In addition, after 4 h of selective REM deprivation, the animals did not show detectable REM sleep rebound (Fig. 1*O*); the REM episode number during the recovery was decreased rather than increased in ZT4–12 (*SI Appendix*, Fig. S2). Overall, *Arc* KO mice had significant attenuation in the homeostatic sleep response to both total SD and selective REM deprivation.

Attenuation of Synaptoneurosome GluA1 Expression in Response to 4 h of Total Sleep Deprivation in *Arc* KO Mice. Next, we investigated whether *Arc* is involved in sleep/wake-associated synaptic homeostasis using an index of GluA1 expression at the synapse and its phosphorylation status, as reported previously (5). We assessed the expression levels of total GluA1 and GluA1 phosphorylated at Ser831 (Ser831P) and Ser845 (Ser845P) across the control, total SD, and recovery conditions. In synaptoneurosomes obtained from the frontal cortex (see *SI Appendix*, Fig. S3), WT mice showed increased expression of GluA1, Ser831P, and Ser845P during SD compared to the control condition, whereas *Arc* KO mice brains did not (Fig. 2 *C–E* and *SI Appendix*, Fig. S4). Using total homogenates, we confirmed the up-regulation of the Arc protein in the brains of WT animals in the SD condition (Fig. 2*I*). In total homogenates, GluA1 protein levels were unchanged across sleep/wake conditions within genotypes (Fig. 2*H*; WT: F [1, 41] = 0.26, P = 0.61; *Arc* KO: F [1, 24] = 0.8345, P = 0.37, two-way ANOVA); however, as reported previously (16), these levels were higher in *Arc* KO mice than they were in WT mice across all conditions (Fig. 2*H*, F [1, 87] = 16.65, P = 0.0001, two-way ANOVA). Interestingly, we found a decrease in *GluA1* mRNA levels after 4-h SD in WT mice, but not in *Arc* KO mice (Fig. 2*J*). Thus, *Arc* KO mice seem to lose the ability to maintain synaptic homeostasis that is associated with GluA1 expression by sleep/wake stimuli.

Impairment of the Induction of Sleep-Related Genes in *Arc* KO Mice. To confirm the importance of *Arc* in sleep homeostasis, we next tested the involvement of *Arc* in the expression of genes previously implicated in sleep homeostasis. As the levels of expression of sleep-related genes in the brain vary in response to the homeostatic sleep drive or to the time spent awake, the loss of *Arc* may affect the expression of those sleep-related immediate early genes (Fig. 3 *A–F*, ref. 11, 30), the expression of genes associated with macromolecule synthesis (e.g., chaperone proteins, Fig. 3 *G–I*, refs. 11 and 31), and energy metabolism (glucose transporter 1, GLUT1, Fig. 3*J*, ref. 11) caused by the disruption of sleep homeostasis observed in Fig. 1. To test the role of *Arc* as a modulator of the expression of sleep-related genes, we compared the expression patterns of already reported sleep-related genes across sleep/wake conditions in the frontal cortex of WT and *Arc* KO mice (11, 30, 31). qRT-PCR data showed that, in WT mice, the expression levels of all these genes were significantly increased after 4 h of SD (Fig. 3 *A–J*) and returned to baseline levels after 2 h of recovery sleep (Fig. 3 *A–I*), except for *homer1a* and *GLUT1* (Fig. 3 *E* and *J*). Importantly, *Arc* KO mice showed a distinct expression profile, especially regarding activity-regulated genes (Fig. 3 *C–F*) except for *BDNF* (Fig. 3*B*): the expression of *c-fos*, *EGR-1*, *homer1a*, *Nur77* (*Nr4a1*), and a chaperone protein, *Bip*/*GRP78*, was not induced after SD and remained at baseline levels after the 2-h recovery sleep (Fig. 3 *C–G* and *SI Appendix*, Fig. S5). These results demonstrate that *Arc* is important for the expression of SD-induced genes, especially activity-regulated genes, after sleep loss.

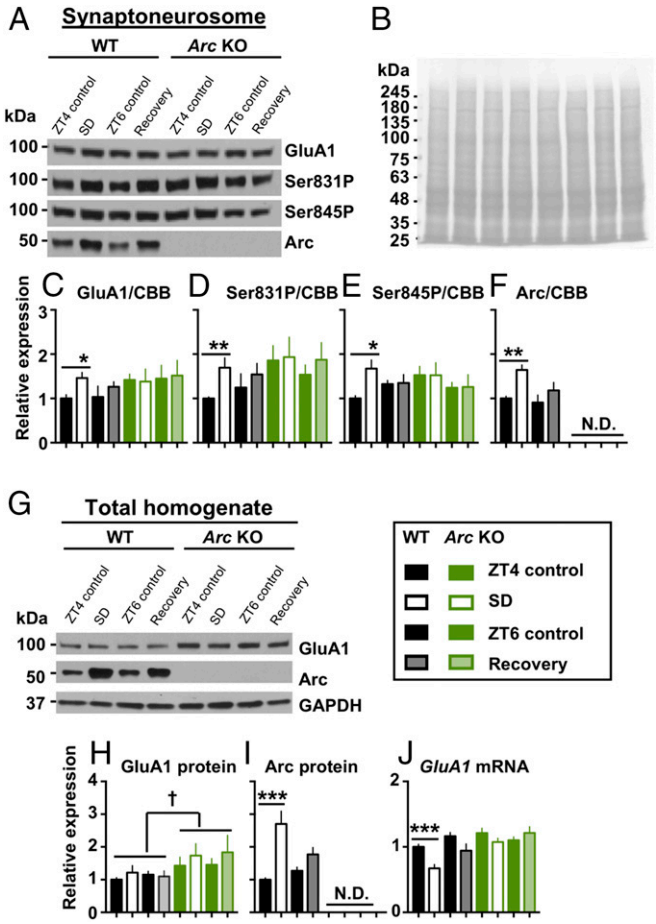

Fig. 2. Impairment of SD-induced synaptoneurosome GluA1 expression in response to sleep deprivation in *Arc* KO mice. Representative images of immunoblots (*A*) regarding GluA1 (*C*), Ser831P (*D*), Ser845P (*E*), and Arc (*F*) in synaptoneurosomes and a transblotted membrane of whole synaptoneurosomes stained with Coomassie Brilliant Blue (*B*). Representative immunoblot images (*G*) of GluA1 (*H*), Arc (*I*), and GAPDH in total homogenates. (*J*). The *GluA1* transcript was measured by qRT-PCR and normalized to *GAPDH* transcript levels. Arc protein expression was not detected (N.D.) in synaptoneurosome (*A* and *F*) or total homogenate samples of *Arc* KO mice (*G* and *I*). Protein expression levels of synaptoneurosome (*C–F*) and total homogenate (*H* and *I*) samples of the frontal cortex were normalized to whole synaptoneurosome proteins and GAPDH in total homogenates, respectively. All values were expressed relative to the means of WT control mice at ZT4. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ by Sidak's multiple-comparisons test following two-way ANOVA; † $P < 0.05$ by two-way ANOVA between genotypes; N.D., not detected (n = 8–14 in each genotype).

Arc Nuclear Distribution in Association with Sleep Loss. It has been reported that the activation of excitatory neurons increases the mRNA (18, 19) and protein expression (19, 20) of Arc, as well as its nuclear translocation (25). Then, in the nucleus, Arc downregulates *GluA1* transcription (25). We confirmed an increase in cellular Arc protein and mRNA expression in the frontal cortex of WT mice as previously reported and also found a decrease in *GluA1* expression in the brains of WT mice after SD; these changes were abolished in the absence of *Arc* (Figs. 2 *I* and *J* and

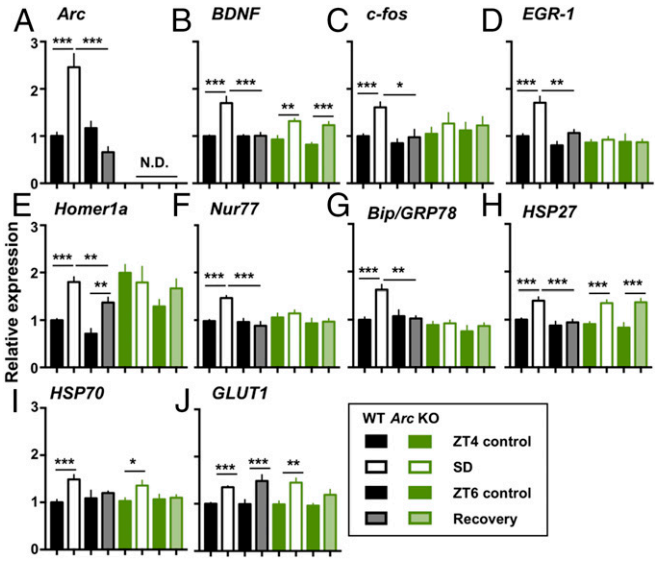

Fig. 3. Distinct expression profiles of sleep-related genes in response to sleep deprivation in *Arc* KO mice. (*A–J*) Expression of SD-induced genes in WT and *Arc* KO mice across the control at ZT4 and ZT6, 4 h of SD, and 2 h of recovery sleep conditions. In contrast to WT mice, *Arc* KO mice exhibited a lower expression profile of SD-related genes in response to SD stimuli (*C–G*). Samples of the frontal cortex were used for the analysis. Gene expression values were normalized to those of *GAPDH* and expressed relative to the means of WT control mice at ZT4 (means + standard error of mean). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ compared to the control condition by Sidak's multiple-comparisons test following two-way ANOVA; N.D., not detected (n = 6–14 in each genotype).

3*A*). These results suggest that SD may lead to the nuclear translocation of Arc (25). Therefore, we investigated the subcellular distribution of Arc protein across sleep/wake conditions in WT mouse brains using immunofluorescence staining.

First, we determined the relative number of Arc-positive cells across sleep/wake conditions in the motor cortex of WT animals (Fig. 4 *A–C*). Qualitatively, we found a unique Arc subcellular distribution that was dependent on sleep/wake conditions, particularly in SD and recovery sleep brains. In 4-h SD brains, Arc accumulated in nuclei (Fig. 4*A SD*, *white arrows*, and Fig. 4*B*). Two hours of recovery sleep following 4 h of SD caused a subset of cells to exhibit the formation of cytoplasmic Arc-positive rings surrounding the nucleus (Fig. 4*A Recovery*, *yellow arrows*); however, these rings were rarely observed in the control and SD brains (Fig. 4*A Controls*, *SD*, and Fig. 4*C*). Immunoblotting performed using nuclear fraction samples supported quantitatively the results of the immunofluorescence experiment; in SD brains, the expression of nuclear Arc was increased by nearly 2.5-fold compared to the control condition at ZT4 (Fig. 4*D*; P = 0.0000007 by Sidak's test) and returned to baseline levels after 2 h of recovery sleep. Similar to the observed SD-associated increase in nuclear Arc levels, SD induced an up-regulation of cytoplasmic Arc by ~2.5-fold compared to the control condition at ZT4 (Fig. 4*E*; P = 0.00009 by Sidak's test). The expression of cytoplasmic Arc was significantly reduced after 2 h of recovery sleep (Fig. 4*E*; P = 0.031 by Sidak's test, SD vs. Recovery). However, in contrast to nuclear Arc, the levels remained higher than the control condition at ZT6 (Fig. 4*E*; P = 0.026 by Sidak's test at ZT6). Taken together, these results demonstrate increases in both nuclear and cytoplasmic Arc levels and in the number of nuclear Arc-positive cells associated with sleep loss.

Discussion

In the present study, we demonstrate that *Arc* KO mice have greatly attenuated homeostatic responses to SD, including the loss of (1) sleep rebound after total SD and selective REM deprivation, (2) SD-induced synaptoneurosome GluA1 expression changes, and (3) induction of a subset of sleep-related

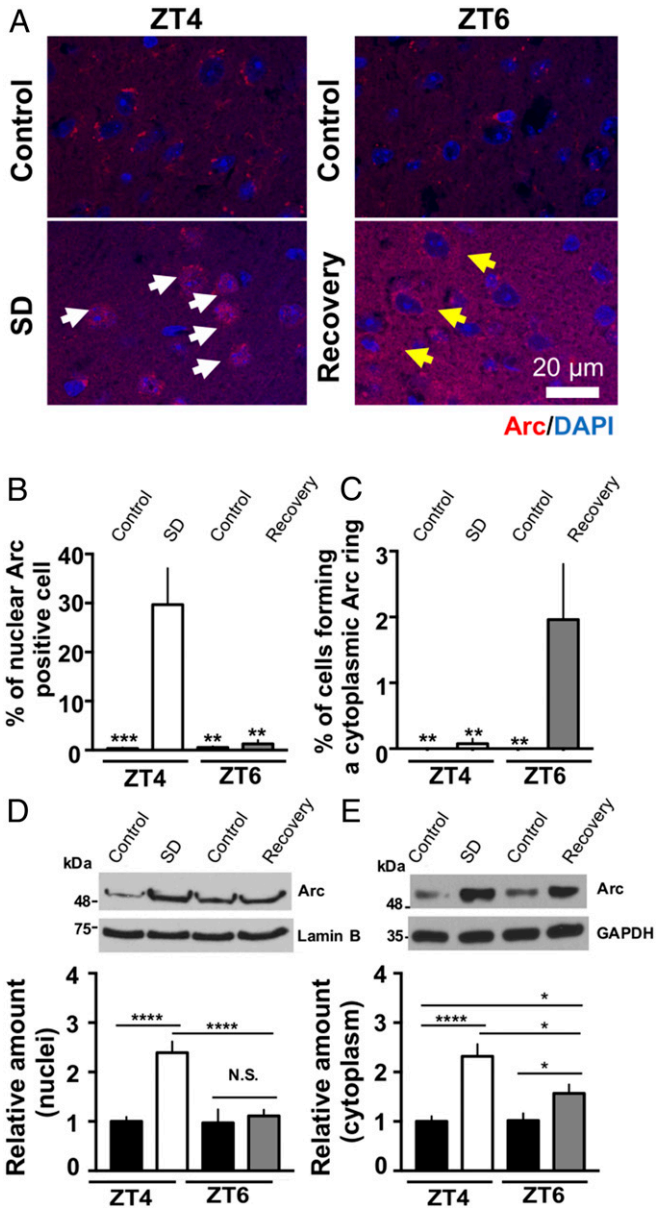

Fig. 4. Subcellular distribution of Arc across the control, SD, and recovery sleep conditions in the brains of WT mice. (*A*) Representative images of the motor cortex (bregma ± 100 μm) for each state. Arc and DAPI signals are indicated in red and blue colors, respectively. White and yellow arrows show representative images of Arc accumulation in the nucleus (SD) and a ring formation of cytoplasmic Arc around the nucleus (recovery), respectively. Percentage of nuclear–Arc-positive cells (*B*) and cells forming a cytoplasmic Arc ring (*C*) in the control, SD, and recovery condition. Arc-positive cells were expressed as percentages of the total DAPI-stained cell (both neurons and glia) numbers. SD and recovery brains showed significant increases in nuclear Arc-positive cells and cells forming a cytoplasmic ring compared to the other groups, respectively (*B*: **$P < 0.01$, ***$P < 0.0001$ compared to SD, *C*: **$P <$ 0.01 compared to recovery, Sidak's multiple-comparisons test, n = 10–15 mice). (*D* and *E*) Evaluation of nuclear (*D*) and cytoplasmic (*E*) Arc expression by immunoblot. The expression levels of nuclear and cytoplasmic Arc were normalized to those of lamin B and GAPDH, respectively, and expressed relatively. *$P < 0.05$, ****$P < 0.0001$ by Sidak's multiple-comparisons test following two-way ANOVA; N.S., not significant (n = 4–9).

genes. In addition, Arc changes subcellular distribution under SD and during recovery sleep. These results are indicative of the multiple roles of *Arc* in the homeostatic sleep response at the behavioral and molecular levels.

Role of *Arc* in Sleep Behavior. The amount of REM sleep is associated with NREM sleep duration (32). A longer NREM sleep duration tends to induce REM sleep. However, *Arc* KO mice tend to have additional REM sleep episodes at the end of NREM sleep episodes more than WT mice do, resulting in a consequent increase in REM sleep amounts (Fig. 1*C*) without changes in a mean duration of NREM sleep episodes under baseline conditions (*SI Appendix*, Fig. S1). Compared with WT animals at baseline, *Arc* KO mice exhibited increases in REM frequencies in the light phase and transitions between NREM and REM sleep, without changes in REM sleep latency. Thus, *Arc* KO mice exhibit a unique phenotype of an increased total time spent in REM sleep. At this time, the mechanism for REM sleep regulation through Arc is unclear. It might be related to the phenomenon that "stress" tends to increase REM sleep (33, 34). Further studies are required to elucidate the Arc-mediated regulation of REM sleep.

It is interesting to note that, after a single exposure to 4 h of SD, *Arc* KO mice showed increased delta power similar to that of WT (Fig. 1 *Q*–*S*), but not increased NREM sleep amount, compared with baseline (Fig. 1*H*). Consequently, NREM delta energy as the integral of delta power density through NREM sleep, which is correlated with a compensation for short-term sleep loss to satisfy sleep need (35), was significantly decreased in *Arc* KO mice compared to WT animals (Fig. 1*W*). Also, in contrast to WT, *Arc* KO mice did not show NREM sleep episode consolidation after 4-h SD (see *SI Appendix*, Fig. S1*F*), suggesting that the animals failed to exhibit this additional index of sleep homeostatic response (36). Moreover, other homeostatic responses including increase of synaptic GluA1 expression (Fig. 2 *C*–*E*) and sleep-related gene induction (Fig. 3) are also blunted in *Arc* KO mice. Thus, we speculate that *Arc* may mediate specific signal(s) through which increased delta power, or sleep need, induces behavioral and molecular changes to maintain normal sleep homeostasis. BDNF has been reported to have a role in the increase of NREM delta power under increased sleep need in humans and rodents (37, 38). Notably, *Arc* KO mice show an apparently intact increase in *BDNF* mRNA levels and delta-power rebound after 4-h SD (Figs. 1*R* and 3*B*). This suggests that BDNF may induce NREM delta-power rebound either through an *Arc*-independent pathway, or acting upstream of Arc signaling. It would be interesting to subject *Arc* KO mice to longer periods of SD; it would also be worth analyzing delta power decay within each NREM episode, which is another index of sleep need (36).

Arc-Mediated Regulation of the Synaptic GluA1 Accumulation. Our results (Fig. 2 *A* and *C*–*F*) demonstrate that *Arc* is important for the SD-induced up-regulation of the GluA1 synaptic pool and of GluA1 phosphorylation (3, 39, 40), which is involved in synaptic strengthening (16–19). In addition, due to the increased GluA1 phosphorylation at Ser831 and Ser845 (16–19, 40), GluA1 surface insertion is likely increased during SD, while decreased in recovery sleep in WT but not *Arc* KO mice (Fig. 2 *A*, *D*, and *E*). Thus, our observations suggest Arc's involvement in synaptic strengthening and downscaling during SD and recovery sleep, respectively. A recent study of the interaction of Arc with PSD-95 shows that this complex is necessary for several physiological postsynaptic functions (41), consistent with Arc's involvement in sleep homeostasis affecting postsynaptic function during SD. Alternatively, a substantial amount of SD-induced synaptic Arc accumulation may be required to induce sleep-related synaptic downscaling during recovery sleep (3, 15, 16, 23). Together, although one of the most important roles of Arc is synaptic downscaling via AMPA receptor internalization at inactivated synapses (23), Arc might contribute to sleep/wake-associated synaptic down/upscaling bidirectionally through the regulation of synaptic GluA1.

***Arc* Involvement in SD-Induced Gene Expression.** As part of the role of Arc in sleep homeostasis, we examined the expression of sleep-related genes and found that Arc was important for the induction of a subset of those genes: the SD-induced expression of *c-fos*, *EGR-1*, *Homer1a*, *Nur77*, and *Bip*/*GRP78* was greatly disrupted (Fig. 3 *C*–*G*), while that of *BDNF*, *HSP27*, *HSP70*, and *GLUT1* was largely unaffected in *Arc* KO mice (Fig. 3 *B* and *H*–*J*). This result leads to three speculations. First, considering that these disrupted genes are all CREB targets (42–46), the CREB pathway or its associated pathways, including calcium-signaling pathways, may be important for the Arc-mediated induction of sleep-related genes. Indeed, the link between Arc and cellular calcium (47), the involvement of Arc in late-phase LTP (22), and the interaction of Arc and CREB-binding protein (48) have been reported. Second, SD-induced genes that are inhibited by *Arc* loss are all immediate early genes, except for *Bip*/*Grp78* (Fig. 3 *C*–*G*). This suggests that Arc may be an important regulator of molecules that are associated with synaptic plasticity via multiple direct or indirect pathways (42–46). Thus, the disrupted expression of sleep-related immediate early genes may also cause the inhibition of sleep-related response of synaptic GluA1 in *Arc* KO mice (Fig. 2*C*). Third, the SD-induced up-regulation of *GLUT1* as a metabolism-related gene or *HSP27* and *HSP70* as chaperone proteins that was normally observed in *Arc* KO mice (Fig. 3 *H*–*J*) suggests that *Arc* is not crucial for the function of sleep related to energy conservation or macromolecule biosynthesis (2). Taken together, these results suggest that *Arc*, itself an immediate early gene, is highly involved in the SD-mediated expression of activity-induced immediate early genes in the brain.

Interestingly, the SD-induced expression of *BDNF*, which is also a target gene of CREB (48), is not affected by the loss of *Arc* (Fig. 3*B*). This seems reasonable because *Arc* is known as a BDNF-induced gene (48). On the other hand, the SD-induced expression of *EGR-1* that also induces Arc expression (49), and a target gene of CREB, was inhibited in *Arc* KO mice (Fig. 3*D*), which likely indicates additional possibilities. Supporting this, the recent report (50) that KCl or light exposure results in a slower induction of *BDNF* compared to the *Arc* or *Fos* genes suggests that, although *BDNF* is an immediate early gene that responds to neural activities, the induction process of BDNF may be differentially activated by extracellular or physiological stimuli, supposedly including SD, compared to other SD-induced immediate early genes we examined here.

Sleep Homeostasis-Associated Arc Translocation between the Cytoplasm and Nucleus. We demonstrated that SD promoted increase in both nuclear and cytoplasmic Arc. Recovery sleep returned nuclear Arc to baseline levels, as assessed by immunoblotting of nuclear proteins together with immunostaining (Fig. 4 *A*, *D*, and *E*). These results fit well with reports that neural activation increases the nuclear import of Arc (25), as the firing rate of glutamatergic neurons increases during waking (51). Notably, cytoplasmic Arc remained higher than baseline levels after 2 h of recovery sleep (Fig. 4*E*), suggesting the possibility that recovery sleep facilitates the cytoplasmic retranslocation of Arc (25). Cytoplasmic Arc are known to be distributed either to the nonsubmembrane cytoplasm or postsynaptic sites (in the soma, dendrites, and spines) through its binding partners (52). SD-induced cytoplasmic Arc may exert spine F-actin formation and reorganization (52) for wake-associated synaptic upscaling, which fits the SHY well (3). A recent histological study reported a sleep-mediated increase in the

nuclear:cytoplasmic ratio of Arc-GFP fusion protein (53), which our biochemical analysis did not positively replicate (Fig. 4 *D* and *E*), perhaps reflecting methodological differences.

Interestingly, the cytoplasmic rings of Arc are not observed at ZT4 (Fig. 4*C*), when total cytoplasmic Arc is high (Fig. 4*E*). Thus, the rings are not associated with increased total Arc in either the nucleus or the cytoplasm (ZT4), but rather with a decrease of nuclear Arc after recovery sleep (ZT6). The total amount of Arc in the cytoplasm (Fig. 4*E*) does not necessarily predict its pattern of distribution within the cytoplasm (e.g., the formation of the ring). The number of cells with a visible ring (only about 2% even at ZT6 after the recovery sleep) probably reflects a small subset of those cells that had high Arc in the nucleus at ZT4 (Fig. 4*B*) and less nuclear Arc after 2 h of recovery at ZT6 (Fig. 4 *B* and *D*). The ring may reflect a transient Arc accumulation just outside of the nuclear envelope in the cytoplasm before it has a chance to disperse evenly into the cytoplasm in association with recovery sleep. Understanding the mechanism for the perinuclear accumulation may be an interesting topic for future studies.

The function of nuclear Arc largely remains to be elucidated compared to that of its cytoplasmic counterpart. Accordingly, little is known about the role of the SD-induced nuclear translocation of Arc. However, in vitro studies have demonstrated that, in the nucleus, Arc down-regulates the *GluA1* mRNA epigenetically through an association with PML-NBs (26). In the nucleus, PML-NBs recruit histone acetyltransferases (HATs)/histone deacetylases (HDACs) (27), implying the possibility that the complex of Arc and PML-NBs may be involved in the bidirectional regulation of downstream gene expression (25, 26, 28). More than 30% of cells exhibited strong nuclear Arc expression in the brains of WT mice in the SD condition (Fig. 4 *A*, *B*, and *D*), which may be involved in transcriptional events of the epigenetically regulated *GluA1* gene. In support of this contention, our results similarly revealed an SD-induced down-regulation of the *GluA1* mRNA in WT (Fig. 2*J*). Moreover, the reduction of nuclear Arc expression level during the recovery sleep (Fig. 4 *B* and *D WT mice*) may cause alterations in the binding affinity of PML-NBs to HATs/HDACs, which may reverse *GluA1* levels to those of baseline in WT brains (Fig. 2*J Recovery*). Furthermore, it was reported that the nuclear Arc-mediated down-regulation of the *GluA1* mRNA consequently reduces the synthesis of the encoded protein, which was observed 30 h after *Arc* transfection in an in vitro study (25). However, in 4- or 6-h WT SD brains, GluA1 protein levels in total homogenates were unchanged (Fig. 2*H*, ref. 5). Supposedly, this time-consuming process of *GluA1* mRNA translation may prevent the detection of any changes in GluA1 protein levels in 4-h SD brains, regardless of its mRNA levels (Fig. 2*J WT mice*). Additional mechanisms for regulating GluA1 translation may also exist in SD brains. Thus, our finding of sleep-related changes in Arc subcellular distribution implies that nuclear Arc may contribute to a mechanism modulating sleep/wake-associated gene expression.

In summary, the present study demonstrates the importance of *Arc* in sleep homeostasis in mice. Arc was induced at high levels in the nucleus, cytoplasm, and synapse during SD and was involved in the induction of sleep rebound, sleep-related gene expression, and GluA1 expression in synaptoneurosomes. Arc is essential for homeostatic sleep regulation at the behavioral and molecular levels, in addition to its role in synaptic plasticity.

Materials and Methods

See *SI Appendix* for detailed materials and methods.

Animals. The animal study protocols approved by the Institutional Animal Care and Use Committee of University of Texas (UT) Southwestern Medical Center were carried out in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals (54). For animal source and husbandry conditions, refer to *SI Appendix, Materials and Methods*.

Data Availability. All data are made available in the paper.

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