Diversity and Mechanisms of State-Dependent Regulation of Synaptic Plasticity

by

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DEDICATION

I dedicate this work to my family, whose sacrifices and unswerving support have made all my accomplishments possible. To my parents, Carlos and Ileana, who left behind their lives in Cuba so that their children could have greater opportunities. To my grandparents, Universo and Ancy Isabel, who soon followed and have been bedrocks of love. To my aunt, Mariuska, whose enthusiasm and selflessness have been constant sources of inspiration. And to my sister, whose tenacity and strong will are enviable. This work is a testament to their love.

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CHAPTER III. How Rhythms of the Sleeping Brain Tune Memory and Synaptic Plasticity

LIST OF ABBREVIATIONS

REM   Rapid Eye Movement

NREM   Non-Rapid Eye Movement

SWS   Slow Wave Sleep

EEG   Electroencephalogram

EMG   Electromyogram

LFP   Local Field Potential

LGN   Lateral Geniculate Nucleus

TRN   Thalamic Reticular Nucleus

V1   Primary Visual Cortex

TC   Thalamocortical

CT   Corticothalamic

SHY   Synaptic Homeostasis Hypothesis

OSRP   Orientation Specific Response Potentiation

LTP   Long Term Potentiation

SD   Sleep Deprivation

PCA   Principal Components Analysis

PSD   Power Spectral Density

FR   Firing Rate

CT0/CT12   Circadian Time 0 / Circadian Time 12

ChR2   Channelrhodopsin-2

ArchT   Archaerhodopsin-T

GFP   Green Fluorescent Protein

TRAP   Translating Ribosome Affinity Purification

ZT0/ZT12 Zeitgeber Time 0/12

HC   Hippocampus

DG   Dentate Gyrus

SWA   Slow Wave Activity

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ABSTRACT

The Synaptic Homeostasis Hypothesis (SHY) posits that a fundamental sleep function lies in regulating synaptic strengths through synaptic weakening. However, the mechanistic details underlying this process, and how extensively it occurs in the mammalian brain, are largely unknown. Studies have focused on specific neocortical regions and excitatory synapses. And it is unclear how inhibitory neurons respond to sleep and sleep loss or whether synapses across all brain regions undergo sleep-dependent weakening. Nevertheless, SHY continues to significantly impact sleep research, with many designing their experiments and interpreting their data under the framework that sleep is a global and uniform process. My thesis work challenges this assumption, working from an overarching hypothesis that sleep dependent synaptic plasticity varies as function of brain region, cell type, and prior experience, and thus pushes the field to consider how sleep may engender divergent plasticity mechanisms across the brain.

In my thesis, I interrogated cell type and region-specific changes in gene expression following sleep deprivation (SD). I used translating ribosome purification (TRAP) to isolate ribosome-associated mRNA from either Camk2a-expressing (excitatory) neurons or parvalbumin-expressing (PV+; inhibitory) interneurons of *ad lib* sleeping or sleep deprived (SD) mice. To look at region-specific changes in transcript abundance after sleep vs. sleep loss, mRNA was isolated from these cell populations in the neocortex and hippocampus. Using quantitative PCR (qPCR), we found significant cell type- and region-specific alterations in immediate early gene (IEG) and clock

transcript abundance following SD. We found hippocampal populations to be substantially less responsive to SD, an effect heightened within parvalbumin cells. We used fluorescence *in situ* hybridization (FISH) on brain tissues from sleeping and sleep deprived mice to quantify changes in IEG expression in parvalbumin (identified by *Pvalb* mRNA expression) and non-parvalbumin (lacking *Pvalb* expression) cells. These results supported the TRAP findings and revealed layer-specific subregional differences in neocortical and hippocampal expression Overall, the data suggests that synaptic weakening across sleep (and strengthening across wake) is a variable phenomenon, dependent on both brain region and cell type.

To test whether slow wave activity (SWA) plays a causal role in sleep dependent synaptic plasticity and visual memory consolidation, I optogenetically stimulated the visual cortices of mice expressing ChR2 in layer 6 corticothalamic neurons (*Ntsr1::ChR2*). Doing so allowed experimental mimicry of SWA in SD mice. I then collected the affected neocortical tissue to determine whether SWA mimicry results in sleep-like activity dependent gene expression. Our preliminary results are inconclusive but promising. While stimulating SD mice constitutively expressing ChR2 blocked SD-driven increases in *Arc* and *Homer1a* expression, we were unable to replicate these findings with AAV-transduced mice. Similarly, while we found that SWA mimicry improved performance of *Ntsr1::ChR2* mice in a sleep dependent visual recognition task, we found no difference in performance between non-stimulated *ad lib* sleep and SD *Ntsr1::ChR2* mice. Nevertheless, SWA mimicry appeared to improve visual recognition, suggesting that SWA mimicry during SD may partially rescue visual memory consolidation.

My thesis work contributes to the sleep field in two significant ways: (1) it challenges SHY's conception of a global and uniform sleep-associated plasticity mechanism and (2) it provides preliminary causal evidence for the role of SWA in sleep-induced gene expression and visual memory consolidation. In challenging this framework, it suggests that future studies should view sleep effects on the brain as inherently heterogenous, opening new avenues of research.

CHAPTER I

Introduction

1.1: The role of sleep in cognition: an unsolved mystery.

Nearly a hundred years of behavioral research indicates a role for sleep in human cognition. Short-term (i.e., hours-long) sleep deprivation (SD) is known to lead to deficits in performance on memory, sustained attention, and perceptual tasks in human subjects [1,2]. Longer-term (i.e., days-long) SD can cause profound cognitive disruption [3]. In animal models, various neurocognitive performance deficits have been described following SD [4,5]. This has led to the hypothesis that at least some forms of synaptic plasticity associated with these cognitive processes occur preferentially during sleep. Recent evidence from both animal models and human subjects has supported this idea. For example, both anatomical [6,7] and functional [8,9,10] remodeling of cortical circuitry after a novel experience occurs selectively during sleep, and is blocked by SD.

Thus for neuroscientists, a critical question is: how does sleep promote nervous system plasticity? Addressing this question has proven difficult. First, as we will discuss here, sleep may promote different forms of plasticity under different environmental circumstances. Thus the effects of sleep (and SD) on the brain may vary with the cognitive

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demands of an animal's present circumstances. Second, the underlying mechanisms driving sleep-dependent plasticity have been elusive. In part, this is because sleep and wake states alter so many aspects of brain physiology simultaneously - neurotransmission, neuromodulation, transcription, translation, neuronal and network activity, interstitial space and ion concentration, etc. [4]

1.2 The synaptic homeostasis hypothesis

What is the synaptic homeostasis hypothesis? Few hypothetical mechanisms have been proposed with an aim toward explaining the many neurocognitive effects of sleep and SD. One notable exception is the sleep and synaptic homeostasis hypothesis (SHY) [11]. SHY has been proposed as an all-encompassing mechanism to explain why cognitive deficits result from sleep loss. SHY proposes that during wake net synaptic strength increases throughout the brain as a function of experience-dependent plasticity; over time this leads to alterations in energy utilization, reductions in space for further plasticity, and disrupted information processing by neurons. SHY further posits that during sleep, synapses throughout the brain are globally reduced in strength (i.e., "downscaled") to offset wake-associated synaptic potentiation. This process is hypothesized to conserve energy, improve the signal-to-noise ratio in neural circuits, avoid saturation of synaptic strength, and prevent pathological levels of excitation in neurons (e.g., epilepsy); it has thus been touted as "the price of plasticity" [12] by proponents of SHY.

Here we discuss SHY in the context of what is currently known regarding the physiology of the brain during sleep. We will review recent data which either support a

SHY-based mechanism for sleep-dependent plasticity, or provide a potential counterpoint to SHY. We also discuss other hypothetical sleep-specific mechanisms which could support brain plasticity.

What is the evidence for sleep-dependent reductions in synaptic strength? Since SHY was first proposed, data to support the hypothesis have come from biochemical [13], electrophysiological [14], and anatomical [15] studies of the effects of brief SD or *ad lib* sleep. These data are outlined in **Table 1** and **Figure 1**, and are described in detail below. *Gene expression:* Early studies that aimed to clarify the functions of sleep in the brain focused on gene expression changes following brief (i.e., hours-long) periods of sleep or SD. These studies assessed changes in mRNA levels in different parts of the brain - hypothalamus [16], neocortex [17,18], cerebellum [17], and hippocampus [19] - using microarray analysis. Across these studies, a consistent finding is that the expression of genes involved in RNA, protein, and lipid biosynthetic pathways, the unfolded protein response (UPR), and synaptic plasticity change as a function of sleep and wake. More specifically, sleep is associated with increased expression of genes associated with protein and lipid synthesis, while SD is associated with increased expression of genes involved in mRNA transcription, cellular stress and the UPR. In support of SHY, in many of these studies, sleep decreases and wake increases expression of a subset of genes thought to be involved in activity-mediated synaptic plasticity - including *arc*, *cfos*, *bdnf*, *narp*, and *homer1a*. More recently, the Allen Brain Institute has made microarray and *in situ* hybridization data available from numerous regions in sleeping and sleep-deprived animals, revealing a more complex picture of gene regulation (i.e., across the entire brain)

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during different behavioral states [20]. These gene expression data have been used as support for the idea that activity-mediated synaptic plasticity is widespread in the brain during wake, and generally reduced during sleep.

Synaptic protein expression: A critical unresolved question is whether the levels of protein translated from sleep- and wake-regulated mRNAs are similarly altered by state. Changes in protein levels appear to track transcript level changes in some of the cases where it has been carefully investigated [21,22]. However, state-dependent changes in protein synthesis [23] may compensate for some changes in gene expression during SD. For example, in the hippocampus, levels of *arc* and *hspa5/BiP* mRNA increase across a brief period of SD; however, due to alterations in translation rates during wake, levels of Arc and Hspa5/BiP protein abundance remain unchanged [24].

Despite these caveats, recent studies have found evidence in support of SHY based on synaptic protein expression. In rats, for example, expression of GluA1 and active (phosphorylated) CaMKII is increased by roughly 20-40% in cortical and hippocampal synaptoneurosomes following a 6-h period of SD, relative to a similar period of sleep [13]. A more recent study [25] reported a similar ~20% decrease in GluA1 and GluA2 content in mouse forebrain synaptosomes during the circadian sleep phase relative to the wake phase. Critically, however, the authors were unable to replicate the previously-reported effects of SD on these targets (i.e., synaptic GluA1 and GluA2 levels were identical with sleep, SD, and SD + recovery sleep). Nonetheless, the authors

concluded based on these data that a global downscaling mechanism acts on synapses during sleep [25].

Synaptic morphology: Effects of sleep have also been seen at the level of dendritic structure in the developing brain. Yang and Gan [26] recently used *in vivo* imaging of layer 5 pyramidal neurons' dendrites in the somatosensory cortex of juvenile mice, to investigate the effects of brief (i.e., 2-h) periods of sleep and SD on spine turnover. The authors found that across 2 h of SD, total dendritic spine/filopodia density increased by ~5%, while across 2 h of *ad lib* sleep, it decreased by ~5%. The difference was apparently due to increased elimination rates for existing spines and filopodia during sleep (there was no difference in the rate of new spine formation between sleep and wake). More recently, serial scanning electron microscopy (SEM) was used to reconstruct and measure dendritic spines (and apposed axon termini) in layer 2 of primary motor and somatosensory cortex of juvenile mice after periods of wake (spontaneous or enforced) vs. sleep [15]. By quantifying the surface area of thousands of individual contacts between axon terminals and spines, the authors concluded that sleep leads to a small (~10-15%) but significant decrease in synaptic contact area. This effect is heterogeneous, with the largest synaptic contacts unaffected by sleep vs. wake. More modest effects of sleep are seen on the size of dendritic spines themselves (e.g. spine volume), with only a small subset of spines quantified showing any effect of sleep vs. wake. While the functional consequence of these changes remains unclear, proponents of SHY have pointed to these findings as the most direct evidence that synaptic strength is reduced during sleep.

Neuronal activity levels and excitatory/inhibitory balance: Numerous recent studies have used neuronal firing rates in freely behaving animals as a surrogate measure for (or potential functional readout of) synaptic strength. While this measure is indirect, and can also be affected by changes in the intrinsic excitability, many laboratories have used it as a potential indicator of overall synaptic strength [9,14,27,28,29]. For example, Vyazovskiy et al. tracked firing rates of rat barrel cortex neurons across periods of sleep and wake, and across the circadian day [14]. In this study, the authors found that neurons tended to fire at a lower rate at the end of the day (when "sleep pressure" - i.e., the propensity to fall asleep - is low) compared with the beginning of the day (when sleep pressure is high). Assuming that firing rate was directly proportional to (excitatory) synaptic strength, the authors concluded that these data indicated that greater synaptic strength is associated with greater sleep pressure, and that sleep reduces synaptic strength (in support of SHY). Importantly however, while these effects on firing rate were present both at the level of multiunit activity and in single neurons identified by the authors as fast-spiking interneurons, they were not statistically significant in putative pyramidal neurons. Nonetheless, this was the first demonstration of a functional change in neural circuits that could be related to the proposed mechanism in SHY.

If we assume (based on these findings) differential effects of sleep on firing in fast-spiking interneurons and pyramidal neurons, one possibility is that excitatory/inhibitory balance (i.e., the ratio of activity in glutamatergic and GABAergic neurons) is the major feature of cortical physiology that changes with sleep pressure. In support of this idea, a recent study of the hippocampal neurons' firing across states found the highest ratio of

interneuron firing -to- pyramidal neuron firing during active wake [30]. Vanini et al. [31] recently demonstrated that the rate of GABA release in the cortex increases steadily across periods of sustained wake. Another recent study found that while glutamate release in rat somatosensory and motor cortex also increases across brief periods of spontaneous wake, with SD, extracellular glutamate levels initially rise (over a period of 30 min- 2 h) and then fall [32]. This supports the idea that sustained wake leads to a gradual decrease in excitatory/inhibitory balance associated with increasing inhibitory neurotransmission.

Synaptic physiology: Additional evidence suggests that synaptic function *per se* may be altered after sleep vs. wake. For example, Liu et al. recently measured the frequency and amplitude of mEPSCs in layer 2/3 pyramidal neurons of juvenile rat and mouse frontal cortex after periods of sleep and wake [33]. While the authors concluded that a 4-h period of SD significantly increased both mEPSC amplitude and frequency, it is worth noting that values for both sleep and wake groups were highly variable. For example, depending on the set of experiments in the study, sleep deprived and sleeping groups' mEPSC frequencies were either quite distinct, or completely overlapped [33]. Furthermore, while frequency changes (presumably reflecting effects on presynaptic release of glutamate) were relatively large, mEPSC amplitude changes (which would be affected by postsynaptic changes in receptor expression) were minimal across sleep vs. wake or SD. However, to date, this is the most direct evidence of functional synaptic weakening across a period of sleep.

Caveats regarding the evidence supporting SHY. The data outlined above has been interpreted by proponents of SHY as evidence of sleep-dependent downscaling, which renormalizes synapses following changes in neural circuits (i.e., synaptic potentiation) caused by wake-associated learning. One major caveat regarding this interpretation is that many of the studies described above (and all of the studies describing anatomical changes) were carried out in adolescent animals (see **Table 1.1**). As is true for humans [34], the rate of synapse elimination in both adolescent rats (corresponding to postnatal weeks 5-9 [35]**)** and mice (corresponding to postnatal weeks 4-8 [36,37,38]) is maximal, and significantly higher than that seen in the adult brain. An unanswered question is whether sleep plays a special role in promoting developmentally-regulated synapse downscaling and elimination in adolescence, or whether sleep-dependent synaptic effects are present across the lifespan. Effects of sleep on synaptic structure and function in the adult brain are still far from clear.

A second caveat is that in many of the studies supporting SHY, comparisons between sleeping and awake animals are confounded by one of two factors. Either 1) samples come from animals spontaneously asleep or awake at different circadian times, or 2) SD animals used for comparison have been deprived of sleep through environmental enrichment (e.g., novel object or running wheel presentation) that was not provided to sleeping animals (see footnotes in **Table 1.1**).

A third major caveat is that while SHY proponents have used a global downscaling mechanism to explain neural network performance improvement using computational

models [39], biological data have not supported the idea of global downscaling during sleep. For example, cortical and hippocampal neurons show non-uniform changes in firing rate across bouts of sleep [30,40]. Specifically, neurons with the highest baseline firing rates (including interneurons) show decreases in spontaneous activity across periods of NREM sleep, while neurons with lower baseline firing rates show either no change, or an increase, in spontaneous firing across a period of sleep. This indicates that functionally, not all neurons are equally affected by sleep, and it stands to reason that not all synapses are equally affected. Indeed, as described above the available anatomical evidence indicates that only a subset of synapses show a reduction in size across a period of sleep [15]. Based on these new findings, the use of the term "synaptic downscaling" may itself be questionable, as sleep does not appear to have truly global effects with regard to reducing synaptic strength (i.e., "scaling" may not be present).

For this reason, more recent descriptions of SHY have proposed that sleep leads to a decrease in the strength *of only a subset* of synapses, while preserving the strength of others. This preservation would be highly desirable for processes involved in learning and long-term memory formation, where information encoded by neural circuits prior to sleep needs to be retained or reinforced. Given these findings, a critical question is why during sleep, some synapses (and possibly some neurons) are apparently unaffected, while others undergo an apparent reduction in strength.

Finally, in none of these studies were the observed changes linked with sleep-dependent cognitive function. The animals under study were housed in standard (i.e.,

non-enriched, and presumably non-challenging) conditions, and were not being trained on specific learning tasks. While sleep affects numerous aspects of cognition (including experience-dependent sensory plasticity and memory consolidation, described in detail below) that are affected by sleep, sleep's effects on these processes have not been linked to synaptic weakening. Thus while converging data suggest that under steady-state conditions, *modest* weakening *of at least some* synapses can been observed in multiple brain areas across periods of sleep, the function of this for information processing in the brain (if any) is still unknown.

What sleep-dependent mechanisms could mediate synaptic weakening? A past major criticism of SHY is the lack of a specific, sleep-dependent, cellular mechanism mediating the observed biochemical and electrophysiological changes [41]. Here we critically evaluate some hypothetical cellular and network mechanisms (see **Figure 1**) for these observations.

Neuromodulatory biasing of spike timing-dependent plasticity (STDP): Recent computational modeling studies from the Tononi lab [42,43] invoked a modified STDP rule to explain reductions in synaptic strength during sleep, and the effects of this process on memory. The STDP rule employed dictated that during learning in the wake state, synapses with temporally correlated pre- and postsynaptic firing would be strengthened, while synapses with non-correlated firing would either be unaffected, or would be weakened. In contrast, during sleep, synapses with temporally correlated pre- and postsynaptic firing would be unaffected (i.e., their strength would be preserved), while

synapses with non-correlated firing would be weakened. In the earlier study, this was implemented computationally by simply inverting the sign of STDP normally seen the cortex [44]. As implemented in this scheme, the same spike timing would cause LTD instead of LTP, for the same pre-post activity pairing, if it was present in sleep instead of wake. The authors argued that the presence or absence of neuromodulators (a function of brain state) would result in the same pattern of firing having differential effects on synapses in the two states. This model was meant to illustrate the benefits of sleep-dependent reductions in synaptic strength, rather than to clarify the cellular mechanisms in operation *in vivo*. However, it is necessary to point out that the proposed cellular mechanism is at odds with neurobiological data in two important ways.

First, sleep and wake are not monolithic with regard to neuromodulation, nor is the neuromodulation state of the cortex binary. Dopamine, serotonin, acetylcholine, and norepinephrine release rates are differentially regulated by state, and these effects vary according to where in the brain release is being measured [45,46,47,48]. Second and more importantly, the effects of the state-regulated neuromodulators dopamine, acetylcholine, and norepinephrine on STDP do not support the notion that STDP rules "flip" between wake and NREM sleep. Each neuromodulator has distinct effects on the relationship between spike timing and synaptic strength changes [49], however, none of these effects fit with the assumptions of the model's modified STDP rule. For example, acetylcholine (with cortical release highest during wake, intermediate during REM and lowest during NREM) [47] can block timing-based LTP and promote timing-based LTD of glutamatergic synapses in cortical pyramidal neurons [50]. In contrast, noradrenergic

signaling (with cortical release highest during wake, intermediate during NREM and lowest during REM) promotes timing-based LTP in both cortical pyramidal neurons and interneurons. These effects are independent of the relative timing of action potentials and EPSPs - i.e., neuromodulator tone, but not the ordering of pre- and post-synaptic activity, determines the outcome of spike pairing. Taken together, available data suggest that higher norepinephrine and acetycholine levels during wake would lower the threshold for inducing *both* STDP-based LTP *and LTD*. It is therefore unlikely that changes in neuromodulation alone would bias plasticity in favor of LTD during sleep.

Homeostatic synaptic downscaling: Central to SHY is the concept of a globally-acting homeostatic mechanism which maintains synaptic strengths within a set physiological range. Homeostatic synaptic downscaling is a mechanism of plasticity that is thought to function in exactly this way, to counteract the network-level effects of excessive neuronal activity and synaptic excitation. Homeostatic downscaling differs from Hebbian synaptic weakening (e.g., LTD) with regard to both mechanism of induction and function. While LTD induction requires appropriately timed pre- and post-synaptic firing, and can lead to functional changes within minutes to hours, homeostatic downscaling appears to require increased neuronal firing and acts over a slower timescale of several hours to days. Homeostatic downscaling was first described *in vitro* by Turrigiano et al. (1998), who described divisive shifts in neurons' mEPSC amplitude distributions in response to long-term increases in firing. Specifically, the authors found that 48 h of exposure to the GABAA receptor antagonist bicuculline led to a global reduction in neurons' mEPSC amplitude distribution (to ~66% of baseline) [51]. This study, along with numerous others

since its publication, have led our current understanding of downscaling, wherein perturbations in either neuronal firing rate or neurotransmission leads to a global reduction of post synaptic strength over several hours to days. Functional decreases in synaptic strength due to downscaling are accompanied by decreases in glutamatergic receptor (e.g. AMPAR) expression and spine volume [51,52,53].

Only recently has sleep been implicated in regulating molecular pathways involved in homeostatic downscaling. Homeostatic reductions in AMPA receptor expression are mediated through multiple cellular pathways, and there is evidence that these pathways may be affected in parallel by sleep. Recent phosphoproteome profiling indicates that a kinase critical for downscaling, cyclin dependent kinase 5 (CDK5), is more active in the brain during the sleep phase of the rodent circadian cycle [25]. CDK5 activity is increased in the nucleus of neurons in response to increased network activity [54], and is implicated in numerous cellular pathways that could promote synaptic downscaling. Within the nucleus CDK5 phosphorylates many targets including MeCP2. This phosphorylation event is critical for decreasing *gluA2* mRNA expression in response to an increase in neuronal activity. CDK5 also interacts with polo like kinase 2 (PLK2) to promote downscaling via downstream effects on the Rap GTPase pathway. This leads to regulation of Rap-mediated changes in AMPA receptor trafficking and dendritic growth [55,56].

A second pathway which has received significant attention as a possible link between sleep and homeostatic downscaling is the Homer1a pathway. *Homer1a* is an

immediate early gene and the short isoform of constitutively active Homer proteins. The constitutive Homer proteins act as scaffolds which bring together a complex including NMDA receptors and mGluR5 receptors at the post-synaptic density. In response to increased neuronal activity, the shorter Homer1a protein acts as a dominant negative isoform, which can disrupt this complex [57]. Loss of Homer1a disrupts homeostatic downscaling [58], and restoring its expression leads to decreased AMPA and metabotropic glutamate receptor expression at the post-synaptic density [59]. Recent gene expression studies have shown that *homer1a* expression increases across the brain in response to SD [18,60] and the genetic locus for *homer1a* has been implicated in the homeostatic regulation of NREM slow wave activity [61]. Diering et al. [25] recently found that Homer1a protein abundance at synapses rapidly increases during SD. If we assume that Homer1a localization at the synapse results in downscaling, this finding would suggest that downscaling occurs during wake. However, the authors also reported that reductions in synaptic GluA1 and GluA2 during the sleep phase of the circadian cycle were dependent on Homer1a. To reconcile these findings, the authors hypothesized that Homer1a mobilization to the synapse is gated by both norepinephrine and adenosine levels. They speculated that during wake, high levels of norepinephrine maintain Homer1a outside the synapse; reduced norepinephrine and increased adenosine levels lead to delivery of Homer1a to the post-synaptic density during sleep. In support of this idea, treating mice with the norepinephrine reuptake inhibitor d-amphetamine (or an A1 adenosine receptor antagonist) reduced synaptic Homer1a levels, while treating them with norepinephrine receptor antagonists increased synaptic Homer1a [25]. The authors of the study argued that this represented a plausible mechanism whereby prolonged

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wakefulness could lead to subsequent synaptic downscaling during sleep. However, it is worth noting that in this study, the observed sleep-associated reduction in GluA1 levels preferentially occurred among spines with the highest baseline levels (i.e., it was not global). Indeed, some spines showed increases in GluA1 levels. Taken together with other evidence showing that synaptic weakening is heterogeneous during sleep [15], these data actually argue *against* true synaptic downscaling as a mechanism for sleep-dependent synaptic changes.

Intriguingly, sleep and wakefulness may have differential effects on so-called "upscaling" - which globally increases synaptic strengths in response to decreased network activity. Hengen et al. evoked homeostatic plasticity in freely behaving mice via monocular lid suture, leading to reduced visual cortex activity. The authors found that homeostatic increases in spontaneous firing rate after this treatment were primarily expressed across bouts of wake, with longer wake epochs resulting in greater firing rate increases [28]. The authors concluded that cellular mechanisms responsible for upscaling are active during wake, and inhibited by sleep. Whether downscaling adheres to this wake/sleep cycle remains unknown but would have important implications for the role of downscaling in SHY.

Although intuitive, homeostatic downscaling as the driver of SHY comes with a significant caveat, it has almost exclusively been studied in-vitro, under conditions that don't reflect wakefulness or sleep. As mentioned above, to induce downscaling researchers have had to create dramatic network perturbations by inhibiting GABBA

receptors for period of up to 48 hours. Whether less protracted and robust activity patterns, reflective of sleep, can induce downscaling is unknown.

Homeostatic maintenance of excitatory/inhibitory (E/I) balance: Numerous studies have indicated that homeostatic responses to increased network activity may also involve modifications to GABAergic synapses, effecting a change in the balance of network excitation and inhibition. Following periods of overactivity, inhibitory synapses on pyramidal neurons have been shown to undergo presynaptic and postsynaptic enhancements, including increases in presynaptic GAD65 and GABAA receptor surface expression [62,63]. Recent data suggest that GABAA receptor surface expression is increased on cortical pyramidal neurons *in vivo* in response to brief SD [64]. Homeostatic increases in GABAA receptor expression have recently been linked to changes in the localization of gephyrin, a scaffolding protein that anchors GABAA receptors to the inhibitory PSD [65]. Flores et al. found that the number and size of gephyrin clusters increase in pyramidal neurons following prolonged network activity. These clusters colocalize with GAD67 and are accompanied by increases in miniature inhibitory postsynaptic current (mIPSC) amplitude and frequency in response to prolonged depolarization of pyramidal neurons. Recent data suggest that this mechanism may be directly affected by sleep vs. wake. For example, gephyrin mRNA levels are higher in the brain after a period of sleep relative to a period of wake [17] Gephyrin is stabilized at the synapse by phosphorylation by CDK5 [66], which as mentioned above may be activated preferentially during sleep [25].

Glutamatergic synapses on inhibitory interneurons may also be potentiated in response to increased network activity, leading to increased feedback inhibition within the network. Chang et al. found that network overactivity results in significantly increased expression of the immediate early gene *Narp* and NARP protein in pyramidal neurons. The authors found that subsequently, NARP is released presynaptically in parvalbumin-expressing interneurons, causing increases in surface expression of GluA4 containing AMPA receptors [67]. *Narp* expression is increased throughout the brain after a period of wakefulness [17]. Given the differential regulation of *Narp* and *gephyrin* expression by wakefulness/sleep, it is possible they maintain network stability by modulating inhibitory activity at different time points to alter E/I balance. Whether these pathways are evoked *in vivo* as a consequence of learning-associated synaptic potentiation is unknown. However, sleep-associated changes in the number of inhibitory synapses have been observed in the cortex, as described above [64]. Taken together, there are numerous alternate pathways by which sleep could regulate homeostatic changes in neural circuits in response to augmented network activity.

NREM oscillation-driven synaptic weakening: Proponents of SHY have speculated that synaptic weakening is mechanistically linked to the synchronous, low-frequency rhythms (slow wave activity; SWA) that synchronize thalamocortical and hippocampo-cortical activity patterns during NREM sleep [68,69]. They argue that, like synaptic strength, SWA is homeostatically regulated. With increased time spent awake (and according to the hypothesis, more opportunity for synaptic potentiation), SWA during subsequent NREM sleep is significantly enhanced. After an initial period of recovery

sleep, this enhanced SWA returns to baseline - a process which is speculated to reflect a renormalization of synaptic strength to levels seen before waking experience. Thus according to SHY proponents, SWA homeostasis and synaptic homeostasis go hand in hand. Beyond this, numerous studies have also indicated that NREM SWA is selectively enhanced in cortical areas that are preferentially activated (e.g., by learning) during prior wake periods. Conversely, SWA is selectively decreased in cortical areas that are less active during prior waking experience [70,71]. In the context of SHY, this has been interpreted as evidence for a causal role of SWA thalamocortical activity patterns in promoting synaptic weakening.

There is evidence that experimentally-generated firing patterns (analogous to those occurring during SWA) can cause LTD of glutamatergic synapses *in vitro*. A variety of paradigms have been used to emulate the activity patterns seen in thalamocortical and hippocampal circuits during NREM. One of these is low frequency stimulation - trains of single spikes or short bursts, occurring at frequencies between 1 and 3 Hz. This rhythmic pattern of activity mimics that generally seen in both hippocampal and cortical circuits during NREM SWA *in vivo*. However, numerous labs have reported that low frequency stimulation (i.e., 1 Hz trains or burst stimuli, which can induce LTD of *in vitro*) is insufficient for *in vivo* LTD induction in either the hippocampus [72,73] or cortex [74,75]. In contrast, higher-frequency stimulation can reliably induce LTP in hippocampal and thalamocortical circuits *in vivo* [76,77,78].

It is unclear why many stimulation protocols induce LTD less robustly *in vivo*, while LTP is more easily induced. In neural circuits where it has been studied, the level of spontaneous activity (which varies with brain state) seems to be a critical variable for both LTD induction and maintenance. For example, LTD can be induced more reliably in the cortex *in vivo* if animals are deeply anesthetized [75]. This effect of anesthesia can be blocked by stimulation of the pedunculopontine (PPT) nucleus (which is wake-active, and provides cholinergic input to the thalamus) [79]. Because PPT activity is generally low during NREM relative to wake [80,81], and because spontaneous thalamocortical activity is generally lower in NREM than in REM or wake [14], it is tempting to speculate that NREM sleep provides ideal (and necessary) state conditions for *in vivo* LTD induction. NREM thalamocortical activation patterns also provide another feature that might be ideally suited for inducing LTD - burst mode firing. Bursts of presynaptic action potentials paired with postsynaptic EPSPs reliably induce LTD of cortical glutamateric synapses *in vitro* [82,83]. Bursts of action potentials with no postsynaptic EPSPs may also reduce subsequent glutamatergic neurotransmission by driving elimination of postsynaptic calcium-permeable AMPA receptors [84]. EPSP-paired bursting can elicit LTD at any time of day (after periods of more sleep or more wake), while unpaired bursting can elicit synaptic depression throughout the day. This suggests that at least two forms of activity-dependent LTD may be expressed at cortical synapses, and these are differentially affected by sleep history. Since these studies were carried out *ex vivo*, and in cortical slices taken from juvenile animals, future studies will have to address how these mechanisms are affected *in vivo* and into adulthood (when rates of synaptic pruning are generally reduced).

There is also evidence that over the long term (24 h, vs. minutes for inducing LTD), low-frequency stimulation may also activate the same cellular pathways involved in homeostatic synaptic downscaling. Goold and Nicoll (2010) recently demonstrated that prolonged optogenetic low-frequency stimulation of individual hippocampal neurons led to both cell-autonomous downscaling of NMDA and AMPA receptor-mediated currents, and dramatic synaptic pruning. These effects were mediated postsynaptically (i.e., in optogenetically-stimulated neurons) via CaMKK and CaMKIV, and removal of GluA2-containing AMPA receptors and NMDA receptors [85].

Despite these data, it is worth noting that NREM sleep is characterized by other network activity features in addition to SWA. In thalamocortical circuits, sleep spindles emerge as 7-15 Hz coherent network oscillations, which are expressed as discrete waxing-and-waning events during NREM [86]. Recent *ex vivo* studies have aimed at mimicking patterns of activity during NREM spindles to determine effects on synaptic strength. Rosanova and Ulrich recorded activity from neurons in somatosensory cortex during spindles, and used this pattern to drive presynaptic activity in layer 2/3 while recording postsynaptic responses in layer 5 [87]. The authors found that when this pattern was repeated at a frequency similar to the frequency of NREM spindle occurrence, postsynaptic responses were potentiated. Moreover, a synthetic spindle activity pattern (presynaptic bursts delivered at 10 Hz) likewise drove postsynaptic LTP. Thus NREM network oscillations of different frequencies may have divergent effects on synaptic strength in cortical circuits.

REM-associated reductions in neural network activity: Proponents of SHY have emphasized the potential mechanistic link between NREM SWA and synaptic weakening. However, most measurements of molecular, functional and structural synaptic changes have been measured after periods of sleep, which includes REM. REM sleep constitutes roughly 10-30% of total sleep time in adult mammals, depending on species. Intriguingly, the proportion of time spent in REM sleep across species has been linked to brain mass [88,89]. Studies evaluating sleep time across phylogeny have not found a similar link between NREM sleep time and brain size. This begs the question - could REM, rather than NREM SWA, mediate synaptic weakening across intervals of sleep? There are some experimental data that would suggest that this is possible. Firing rates in both cortical [8,40] and hippocampal neurons [30,90] decrease consistently across bouts of REM. Firing decreases are proportional to REM bout duration in the cortex [40] and to the amplitude of locally-generated theta (4-12 Hz) oscillations in the hippocampus [30,90]. A recent fMRI study [91] indicated that overnight decreases in amygdala functional responses to an emotionally arousing task are related to REM-associated EEG activity. More recently, a study measuring overall levels of cortical neural activity (with wide-field imaging of calcium signals) found that activity is globally reduced in the cortex (in all cortical layers) across bouts of REM [92]. In support of the idea that these functional changes are related to synaptic weakening, a recent *in vivo* imaging study demonstrated that the selective elimination of newly-formed dendritic spines is blocked by REM-targeted SD (but not NREM disruption) [93]. What features of REM could mediate synaptic weakening? Recent calcium imaging data indicates that the relative activity of fast spiking

interneurons to pyramidal neurons is significantly higher during REM relative to NREM and wake [92]. Thus REM may alter the E/I balance of neural networks, which could bias plasticity at glutamatergic synapses, to favor synaptic weakening. Alternatively, the relative high levels of cortical and hippocampal acetylcholine release (and simultaneous relative low levels of norepinephrine, serotonin and dopamine release) during REM may bias circuit plasticity in favor of spike timing-based LTD (see above).

Glial regulation of synaptic function: Multiple lines of evidence have indicated that the biological support system surrounding neurons is significantly affected by sleep and wake states. Recent studies focused on the so-called "glymphatic" system have shown that interstitial space in the cortex increases significantly during NREM sleep, over a timescale of minutes [94]. This process, mediated by astrocytic regulation of peri-arterial flow rates, is thought to promote both delivery of nutrients, and clearance of potentially harmful metabolic waste from the brain. Such a mechanism could affect synaptic function in myriad ways. For example, levels of extracellular glucose decline across bouts of wake and REM, and increase at the transition from wake to NREM sleep [95]. At the same time, lactate accumulates in the brain (as a product of glycolysis) during wake (and also during REM sleep) [96] and is cleared by the glymphatic system during NREM sleep [97]. Because at high enough concentrations lactate can potentiate NMDA receptor-mediated currents, leading to downstream changes in the expression of plasticity-related genes in the brain [98], this mechanism could potentially mediate sleep-dependent synaptic weakening.

Sleep changes not only the volume of the brain's interstitial space, but also its ionic content. A more recent study demonstrated that the extracellular concentrations of calcium, magnesium, and protons increases (and the concentration of potassium decreases) in the cortex as animals transition from wake to NREM sleep [99]. As might be expected, such changes directly impact the mode of firing in cortical neurons (and ECoG activity), but it remains unclear whether they also directly impact synaptic function and synaptic strength. Available data suggests that changes in the concentrations of these ions (like those that accompany wake-to-sleep transitions) can lead either to a selective increase in excitatory transmission (resulting in increased E/I balance) [100] or to synaptic potentiation [101,102,103,104]. Intriguingly, these extracellular ion concentration changes could all be mediated by astrocytes [105] and could in turn impact the activity pattern of surrounding neurons [99]. Indeed, recent experimental data has shown that optogenetic hyperpolarization of astrocytes leads to changes in local field potential (LFP) activity similar to that seen as animals transition to NREM sleep [106].

While the precise cellular mechanisms underlying all of these effects are generally unknown, it is clear from studies using cell type-specific mRNA profiling (i.e., translating ribosome affinity purification; TRAP) that sleep and wake affect a variety of cellular processes in both astrocytes [107] and oligodendrocytes [108]. One speculative mechanism is based on the fact that ATP released from neurons during heightened network activity activates purinergic receptors on microglia, leading to release of interleukin 1 (IL1) and tumor necrosis factor-alpha (TNFα) [109,110]. Because IL1 and TNFα can induce NREM sleep, this signaling mechanism has been hypothesized to
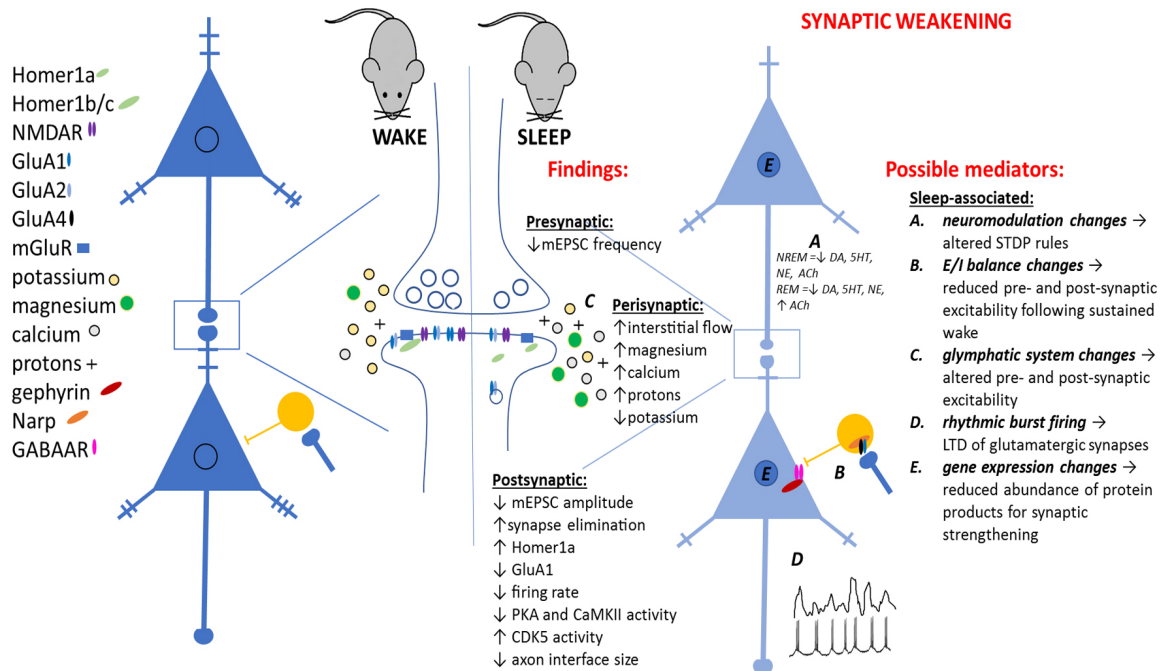
Figure 1.1. Observed pre- and post-synaptic changes attributed to sleep-dependent synaptic weakening, and potential sleep-dependent mechanisms.

mediate both sleep homeostasis after extended wake, and local, use-dependent changes in NREM thalamocortical oscillations [111]. Some have speculated that this same signaling pathway may also mediate sleep-associated synaptic weakening. However, because the *in vitro* effects of TNFα on glutamatergic [112,113] and GABAergic [114,115] synapses are diverse, it remains unclear whether glial-derived TNFα signaling offers a plausible molecular mechanism for synaptic weakening during sleep.

1.3 A counterpoint to SHY - a role for synaptic strengthening in the cognitive benefits of sleep

An increasing body of data has presented counterpoints to SHY (see **Table 1.1**). These studies have primarily been aimed at investigating the neurobiological correlates of sleep-dependent learning and memory storage, following novel learning experiences during wake. Surprisingly, many of these investigations have found evidence of synaptic strengthening, not weakening, across periods of post-learning sleep (see **Table 1.1** and **Figure 1.2**). Thus one possibility, which we put forth here, is that different types of synaptic plasticity (not synaptic weakening alone) may be promoted during sleep, depending on the circumstances of an animal's prior waking experience. Here we will briefly describe what is known about some example cases in which synaptic strengthening occurs during sleep, leading to adaptive changes in brain function.

Contextual fear memory (CFM): CFM is a well-studied form of long-lasting memory, which can last days or even months in mice following a single learning experience. As such, it has been described as analogous to episodic memory in humans. CFM

consolidation is disrupted by SD in the hours following single-trial contextual fear conditioning (CFC) [116,117]. CFM consolidation relies on neural activity in hippocampal area CA1 during the same window of time post-CFC [118]; a reasonable conclusion is that network activity patterns in CA1 during sleep play an essential role in memory storage. Recent work from our lab [119] has demonstrated that during this window of time, CA1 neuronal firing and the amplitude of network oscillations are enhanced; these changes from baseline are present during both NREM and REM sleep. Furthermore, functional connectivity relationships between CA1 neurons (quantified based on relative spike timing among stably-recorded neurons) are selectively enhanced during NREM sleep following learning. This change is present across NREM over the entire 24 hours between CFC and fear memory testing - suggesting a plausible neural substrate for memory storage. More recently, we found an experimental strategy to disrupt the post-CFC enhancement in NREM and REM CA1 oscillations - by selective inhibition of parvalbumin-expressing (PV+) interneurons in the hours following CFC. We found that pharmacogenetic inhibition of PV+ interneurons disrupts both stabilization of functional connectivity patterns in CA1 during NREM, and CFM consolidation [120]. By optogenetically activating PV+ interneurons in a rhythmic fashion (mimicking rhythms enhanced during post-CFC sleep), we were able to both stabilize and strengthen functional connectivity relationships between neurons across CA1. Taken together, this suggests that sleep oscillations which are augmented in the hippocampus after learning promote long-term memory formation via synaptic strengthening, rather than synaptic weakening. CFM consolidation is linked mechanistically to LTP of glutamatergic synapses in CA1, for several reasons. First, behavioral manipulations such as SD that interfere with

CFM consolidation also disrupt Schaeffer collateral LTP in CA1 [121]. Second, disruption of intracellular pathways required for LTP in CA1 also disrupt CFM consolidation [7,121,122,123]. Third, intracellular pathways required for LTP are activated in the hippocampus immediately following CFM [122]. Finally, experimental manipulations that enhance hippocampal LTP also enhance CFM consolidation [124]. Thus all available evidence suggests that in the case of CFM consolidation, sleep activates cellular pathways in the hippocampus to induce synaptic potentiation (not downscaling), in order to promote memory formation. Taken together, these data present a clear non-SHY synaptic mechanism underlying specific cognitive benefits of sleep.

Motor cortex plasticity after learning: A large number of studies using human subjects have shown benefits of sleep for sensorimotor performance following learning a new sensorimotor task [125,126]. Depending on the specific motor task involved, these studies have linked the benefits of sleep on motor performance to changes in local slow wave and spindle oscillations in supplementary motor cortical areas following learning [127], changes in SWA in parietal cortical areas involved in multisensory spatial information processing [70], and total post-learning NREM sleep time [128]. Additionally, experimental disruption of cortical SWA following learning has been shown to disrupt consolidation of at least some forms of sensorimotor learning [129].

Recent studies using repeated functional brain imaging during motor task acquisition demonstrated that a correlate of sleep-dependent performance enhancement is an increase in task-related brain activity in corticostriatal and cerebellar motor systems

following a period of sleep [130,131]. This increase in task representation in the brain after a period of post-learning sleep is suggestive of synaptic strengthening, insofar as BOLD signal changes reflect changes in the extent of synaptic activity. A more definitive demonstration of sleep-dependent synaptic strengthening (or at least synaptic growth) during NREM sleep occurs following motor learning in mouse primary motor cortex (M1) [6]. In their recent study, Yang and colleagues demonstrated that SD (but not REM-selective SD) disrupted formation of new dendritic spines in M1 layer 5 (i.e., output) pyramidal neurons in the hours after a period of motor learning.

Ocular dominance plasticity (ODP) and orientation-specific response potentiation (OSRP) in the visual cortex: There are multiple examples of synaptic strengthening during sleep from the visual system following novel visual experiences. One is the effect that sleep has in the primary visual cortex (V1) in the context of ocular dominance plasticity (ODP) - a well-studied form of cortical response plasticity initiated by loss of visual input to the cortex from one of the two eyes. ODP is an adaptive response that shifts V1 neurons' visual responsiveness from binocularity to favoring the spared eye. The role of sleep in promoting this process has been studied for nearly two decades. In 2001, Frank et al. initially reported that during a sensitive period of postnatal development, a modest shift in visual responses occurs in cat V1 following a brief (6-h) period of monocular visual experience [132]. This shift is effectively reversed by a subsequent 6-h period of SD (without visual input) but is significantly augmented by 6 h of subsequent *ad lib* sleep. The mechanism mediating this sleep-dependent enhancement of ODP involves activation of LTP-mediating kinase pathways, relies on NMDA receptor activation and

protein synthesis, and causes an enhancement of V1 neurons' firing rate responses to spared-eye stimulation [10,133,134]. These changes are associated with changes in V1 network activity during sleep in the hours following monocular experience - including reduced fast-spiking interneuron firing (which occurs specifically in cortical areas representing the spared eye), increased principal neuron firing, and increased neuronal firing coherence with both slow wave and spindle oscillations in NREM sleep [9,10].

While ODP 1) is induced by a loss of visual input, and 2) is most robustly induced during a relatively brief postnatal window, orientation-specific response potentiation (OSRP) is expressed in adulthood in mouse V1 in response to specific patterns of visual input [135]. Our laboratory has shown that following a brief period of exposure to an oriented grating stimulus (lasting 30-60 min), OSRP is expressed in V1 neurons as an enhanced response to stimuli of the same orientation. This response change is not present immediately following the visual experience, but is only seen after 6-12 h of subsequent sleep [8,27]. OSRP is blocked by post-stimulus SD, and is proportional to post-stimulus NREM and REM sleep time [8,27]. As is true for both CFM consolidation and ODP in V1, OSRP consolidation is associated with an increase in firing rate among V1 neurons in the hours following experience (which apparently occurs across bouts of NREM and REM, not wake) [8], and is proportional to an increase in phase-locking of V1 neuronal firing to NREM oscillations [27]. The expression of OSRP is linked to clear, stimulus-selective enhancement in firing rate responses to visual stimulation in V1 neurons, suggestive of synaptic potentiation [8]. This interpretation is consistent with studies of the underlying mechanisms of OSRP. For example, OSRP is blocked by

interference with cellular pathways required for LTP of glutamatergic synapses [135]. Further, *in vivo* thalamocortical LTP induction (with high-frequency LGN stimulation) occludes subsequent induction of OSRP, and OSRP induction occludes subsequent LTP between LGN and V1 [78]. Together, these data suggest a common mechanism between LTP of thalamic relay synapses in the cortex and sleep-dependent OSRP consolidation.

A data-driven alternative to SHY: What do all of these exceptions to SHY have in common? In all cases, the animal is being trained on a novel task, or having a novel experience, immediately prior to sleep. Based on available data, we propose an alternative to SHY - an alternative that applies to situations where sleep follows a learning experience in wake. In this scenario, we propose that circuit-specific changes in gene expression and protein translation during wake lead to subsequent changes in network activity during subsequent sleep. These changes in network activity support strengthening of at least a subset of network glutamatergic synapses (see **Figure 1.2**). We speculate that, consistent with the examples described above, sleep-dependent synaptic strengthening is essential for the cognitive benefits of sleep. In contrast (and in contradiction to SHY) sleep-associated synaptic weakening may not play a critical role in promoting cognitive function. Thus far, there is scant evidence to suggest that sleep-dependent learning and memory processes are related to synaptic weakening, and none to suggest an association with homeostatic downscaling.

The forms of sleep-dependent plasticity described above have several features in common. They are all associated with circuit-specific changes in network activity

including: 1) increases in neuronal firing rate, 2) amplified NREM (and occasionally, REM) oscillations, and 3) phase-locking of neuronal firing to these oscillations. Current data suggest that these changes are the direct result of learning experience during prior wakefulness. We speculate that while synaptic weakening may occur across sleep in the absence of learning (e.g., for mice housed in standard conditions), post-learning changes to network activity in the sleeping brain can support synaptic strengthening.

Synaptic strengthening in NREM sleep: SHY proponents have linked synaptic weakening during sleep to NREM oscillations. In the cases described above, however, NREM oscillations (and neuronal firing coherence with them) have been linked to synaptic strengthening and growth, resulting in memory consolidation, adaptive sensory plasticity, or motor learning. Might NREM oscillations differentially affect synaptic strength (bringing it either up or down within a given circuit) depending on prior experience? This is a possibility. Indeed, work from our own lab suggests that this may be the case. One example of this is the firing rate changes that occur in individual V1 neurons after a period in of dark exposure (i.e., no visual experience) vs. patterned visual experience. In the former case, an increase is seen across bouts of wake, no change across NREM bouts, and a decrease across REM bouts; in the latter, firing rates increase selectively during NREM and REM sleep bouts (but not wake). Another example comes from the rat somatosensory cortex, where prior experience with a spatio-tactile task (novel object exploration) led to selective increases in firing rate during the next 3 h of subsequent sleep [136].

The idea that NREM oscillations play a critical role in patterning brain plasticity was recently reinforced by findings from a study using optogenetics to mimic NREM slow wave oscillations (with simultaneous 2 Hz stimulation of mouse somatosensory and motor cortex) following training on a somatosensory perceptual task [137]. The authors found that synchronous stimulation of the two areas rescued perceptual learning in mice from deficits induced by post-learning SD. Chauvette et al. [138] recently attempted to clarify the immediate effects of NREM slow wave oscillations on synaptic strength in the cat cortex, *in vivo* and *in vitro*. The authors found that cortical evoked potentials were enhanced selectively across periods of NREM sleep (but not across periods of wake or REM). They also found that presynaptic stimulation patterned to mimic that seen in SWA (but not stimulation patterned to mimic wake activity) led to long-term increases in EPSP amplitude in cortical neurons. A more recent study [139] showed that in the hippocampus, neuronal firing in the context of a sharp wave ripple oscillation can directly promote LTP *in vitro*. A reasonable conclusion is that the firing patterns evoked by NREM oscillations are conducive to synaptic potentiation.

Synaptic strengthening in REM sleep: The majority of recent work focused on sleep-dependent plasticity has emphasized a role for NREM sleep in the process. However, it is worth noting that a number of findings have suggested that synaptic strengthening can occur specifically in REM sleep. For example, either brief (i.e. hours-long) or long term (days-long) periods of REM-targeted SD, can disrupt subsequent induction and maintenance of hippocampal CA1 LTP [140,141]. Related to this deficit, brief REM-targeted SD in the hours following learning is sufficient to disrupt some forms of

hippocampally-mediated memory consolidation [140,142]. These effects are related to changes in PKA and CREB signaling, and changes in the expression of Arc and BDNF, in the hippocampus and in other areas involved in mnemonic processing [143,144]. While the systems- and network-level mechanisms responsible for REM's influence on hippocampal LTP and hippocampally-mediated memory formation are still largely unknown, memory consolidation in some REM-dependent tasks is correlated with the occurrence of pontine-geniculate-occipital (PGO) waves (which occur preferentially at the transition from NREM to REM and during REM). Activation of pontine circuitry that promotes PGO waves (leading to increased PGO wave occurrence) can rescue certain forms of REM-dependent memory in the context of REM SD [145]. More recently, REM sleep was also shown to play a critical role in the consolidation of ODP in cat V1. The shift in visual responses in favor of the spared eye was greatly reduced when REM sleep was selectively deprived in the hours following monocular visual experience [146]. REM SD also disrupted visual experience-induced enhancements in LTP-mediating kinase (i.e., ERK) activity in V1 during post-experience sleep. Furthermore, neuronal firing rates are increased during post-learning REM, in both mouse hippocampus in the hours after single-trial CFC [119,120], and in mouse V1 following induction of OSRP [8,27]. Indeed, changes in firing rate in V1 neurons increase more across bouts of REM than across bouts of NREM in the hours following novel visual experience [8]. These changes, like changes in the occurrence of PGO waves, and the expression of many immediate-early genes involved in synaptic potentiation, are dependent on experience during prior wake [8,27,119,144,147].
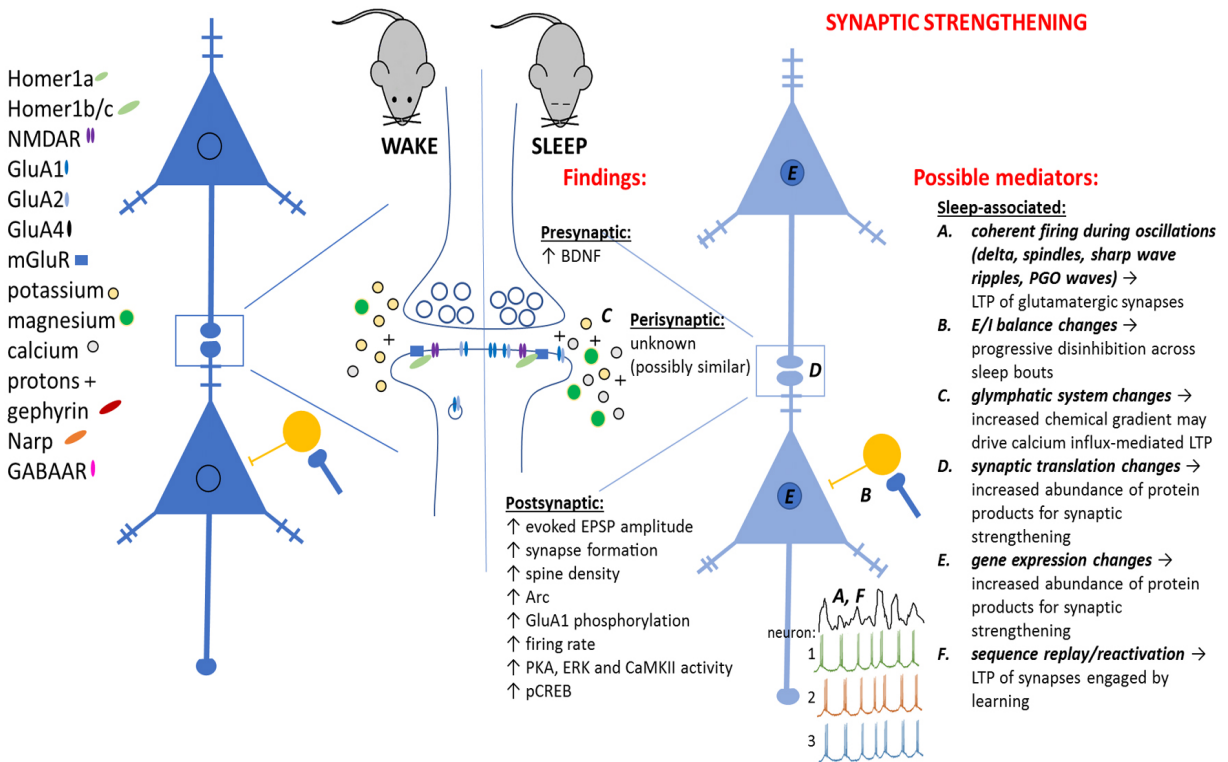
Figure 1.2. Observed pre- and post-synaptic changes attributed to sleep-dependent synaptic strengthening, and potential sleep-dependent mechanisms.

1.4 The function of sleep-dependent "replay" of network activity patterns

What is replay? A great deal of recent data suggest that reactivation of task-associated neuronal ensemble activity patterns occurs during subsequent sleep, leading to speculation that this reactivation drives sleep-dependent memory consolidation. One barrier to our understanding of the function of reactivation in neural circuits is that it has been defined using a variety of conceptual and quantitative means. Early studies by Pavlides and Winston [148] defined task-associated activity as temporally-correlated firing among neuron pairs during experience. Using this definition, Pavlides and Winston (and others) first described sleep-dependent reactivation of place cells following exploration of new environments [148,149]. Other recent studies have described stabilization of functional communication patterns (based on spike timing between neurons) during NREM sleep following single-trial learning [119,120] or selective reactivation during REM sleep of neurons activated by novel sensory experience [146]. Because such network-level changes occur during sleep following a single learning event, they are plausible substrates for promoting synaptic plasticity.

In recent years, however, the term "replay" has been used in reference to precise sequential reactivation of neurons engaged sequentially during a spatial task. For technical reasons, the majority of these studies have focused on the reactivation of hippocampal place cells — neurons with spatially selective receptive fields. As an animal traverses an environment, place cell neurons fire to encode its changing location, creating sequential patterns of activation that reflect its trajectory. Using this sequence as a template, one can quantify replay events during subsequent REM or NREM sleep

[150,151]. An essential component of experiments measuring sleep-associated sequential replay is the generation of a reliable, repeatable behavioral sequence. In studies using rodents, this usually requires weeks of repetitive training on a spatial task. Because the animals in these studies are carrying out a familiar (rather than new) task prior to measurements of replay events, the relationship between sequence reactivation and new memory formation is not generally clear.

What causes replay? How does sequential replay occur? One parsimonious interpretation of data involving highly trained animals engaged in a repetitive spatial task is that the sequence of neuronal activation is simply "hard-wired" due to the strength of connections between neurons in the ensemble. This might explain the fact that replay, relative to sequential activation during behavior, tends to be time-compressed. If neurons in the ensemble were synaptically connected and played a strong causal role in driving one another's firing, they would fire sequentially during spontaneous activity with minimal synaptic delays. It would also explain the fact that replay events can occur in practically any brain state (with reports of replay in NREM, REM, and wake)[152]. Finally, if the neurons were reciprocally connected, this interpretation could also explain the occurrence of reverse replay events (where the sequence of neuronal activation is opposite that seen during behavior) [153]. A related mechanism that has been proposed (the so-called "lingering excitability model") [154] is based on the relative excitability of place cells, where neurons that have been most recently activated (i.e., by the animal's recent presence in their respective place fields) are more likely to initiate a sequential (forward or reverse) replay event. This would explain the apparent hysteresis of replay events. For

example, sequences of activity that have occurred more frequently in an animal's recent past (during behavior) are more likely to replay when the behavior ends [154]. Furthermore, during pauses in a run, replay sequences are most likely to initiate with the firing of the place cell representing the space that the animal currently occupies [154]. However, neither of these explains another phenomenon related to replay - the occurrence of sequential activity patterns *before* a set of place cells is sequentially activated during behavior (so-called "preplay"). Preplay maps of place field activation have been reported to predict future trajectories, *despite occurring prior to actual experience*. Recently, a study by the Foster lab questioned the occurrence of preplay events, suggesting that they may result from a statistical anomaly. Using a larger sample of neurons, and slightly different quantitative methods, the authors were unable to find evidence of preplay events [155]. Nonetheless, reports of reverse replay and preplay, which can at times represent never before experienced behavioral sequences, brings into question the hypothesis that replay promotes memory consolidation.

Does replay play a role in memory consolidation? Despite the caveats outlined above, various arguments have been put forward in support of the idea that sequential replay could promote memory consolidation, particularly in the context of post-learning sleep. During replay events, sequential patterns of neuronal activation are compressed to a time scale compatible with STDP. Such compressed replay occurrences preferentially occur during sharp wave ripple events, which 1) occur preferentially in the hippocampus during NREM sleep and 2) have themselves been linked to memory formation [156]. Thus it has been argued that replay offers an instructive mechanism for promoting formation of

specific memories, by altering the strength of connections between neurons sequentially engaged during waking experience. Coordinated replay between brain areas (typically hippocampus and cortex) during sleep is proposed to be a critical mediator of systems memory consolidation [4,157]. Sequential replay of neuronal activity patterns has been seen in cortical structures like the prefrontal cortex following spatial task performance [158], and coordinated hippocampal and cortical sequential replay has been described in the context of spatial learning (i.e., maze running)[150]. However, there is currently no evidence that such sequence reactivation is temporally associated with, or critical for, *de novo* memory formation. In contrast, there are suggestions that sleep-dependent, coordinated reactivation of specific neuronal populations in hippocampus and cortex may promote information transfer between the two structures. For example, a recent study using dual-site recording found that NREM sharp wave ripple events in hippocampus triggered reactivation of neuronal ensembles in prefrontal cortex that were co-activated during prior spatial task learning [159]. Intriguingly, while early data suggested preferential information flow from hippocampus to cortex during NREM sharp wave ripple events [160,161], more recent findings suggest that activity patterns in the cortex can inform the activation pattern in the hippocampus during these events [162]. Because during NREM, hippocampal sharp wave ripples are coordinated with neocortical slow waves [68,163], this suggests that during NREM-associated oscillations there is a true dialogue between neurons in hippocampal and thalamocortical circuits. Such a dialogue may promote the formation of widely distributed memory traces in the context of consolidation.

A major unresolved question for the field is whether replay or reactivation promotes synaptic plasticity and long-term memory formation. Because the memories in question are associated with activity in sparsely-distributed neuronal populations, direct measurement of functional connectivity (i.e., mEPSC amplitude or frequency) or anatomical plasticity (i.e., spine size or density) associated with memory consolidation is a technical challenge. A few studies have attempted to resolve whether replay events can be disrupted by NMDA receptor antagonism *in vivo* [155,164]. These studies are illuminating for multiple reasons. First, such antagonism is almost universally amnestic for the types of (episodic or spatial) memories typically under study with respect to replay. Second, since many forms of Hebbian plasticity rely on NMDA receptor signaling, this treatment should disrupt any events relying on, for example, LTP. Data from these studies suggests that replay/reactivation events related to newly-learned trajectories or locations is lost in the absence of NMDA receptor signaling [155,164]. This suggests that replay occurrence is at least related to new memory formation.

1.5 Thesis Goals and Summary

It is clear that sleep is critical for memory consolidation and increasingly more studies suggest that it facilitates the expression of several plasticity mechanisms. However, how sleep engenders the changes described above remains unclear. And, whereas sleep is often viewed as a unidirectional and globally uniform phenomenon, several studies suggest that there is significant heterogeneity in sleep-dependent processes. In this thesis, I investigate cell and region-specific responses to sleep loss and present preliminary data assessing the role NREM slow wave activity in sleep-dependent

weakening. In addition, I review the current literature on neuronal oscillations during sleep and present a theory of how oscillations may enact plasticity mechanisms during sleep. These goals are addressed in the chapters outlined below.

Chapter II: Sleep loss drives brain region- and cell type-specific alterations in ribosome-associated transcripts involved in synaptic plasticity and cellular timekeeping

In this chapter I use translating ribosome affinity purification (TRAP) to isolate Camk2a+ and Parvalbumin+ neurons from the neocortex of sleep deprived (SD) and sleeping mice. I then interrogate how SD impacts the ribosome associated transcript abundance of particular immediate early genes and clock genes. I confirm these results and quantify additional regional differences in SD driven gene expression with fluorescence *in situ* hybridization.

Chapter III: How rhythms of the sleeping brain tune memory and synaptic plasticity

In this chapter I provide a thorough overview of what we know regarding sleep oscillations and their role in sleep-dependent synaptic plasticity and memory consolidation. I also present a unifying theory of oscillatory mediated synaptic plasticity.

Chapter IV: Effects of NREM slow wave activity on neocortical gene expression and visual memory consolidation

In this chapter I interrogate the role of SWA in sleep dependent synaptic weakening by optogenetically mimicking this oscillation in sleep deprived mice. I then compare the expression of several immediate early genes from SD, ad lib sleep, and

SD with stimulation mice to determine whether SWA is sufficient in blocking SD-driven

increases in IEG expression. Using this experimental paradigm, I then test whether

mimicking SWA is sufficient to rescue performance in a sleep dependent visual

recognition task.

Table 1.1. Summary of evidence in support of sleep-associated synaptic weakening, and sleep-associated synaptic strengthening.

<div align="center">Evidence for synaptic weakening during sleep:</div>

Manipulation	*Key findings*	*Species*	*Age*	*Brain area*	*Reference*
Biochemistry:					
5 h sleep vs. SD[1]	induction of *arc, fos,* and *creb* during SD	mouse	2-4 months	hippocampus	[19]
3-12 h sleep vs. SD[1]	*narp, fos,* and *bdnf* induced during SD in cortex	mouse	10 weeks	cortex (somatosensory and motor) and hypothalamus	[18]
sleep (ZT8), 8 h SD[2] (ending at ZT 8), wake (ZT20)	during wake and SD, *bdnf* and *narp* induced in cortex and cerebellum; *homer1a, fos,* and *arc* induced in cortex	rat	unknown	cortex (unknown areas) and cerebellum	[17]
sleep (ZT6), 6 h SD[3] (ending at ZT 6), wake (ZT18)	during wake and SD, ~20% increase in GluA1, pCaMKIIa, and pSer845-GluA in synaptoneurosomes from both areas	rat	12-14 weeks	cortex (unknown areas) and hippocampus (synaptoneurosomes)	[13]
sleep (ZT4), 4 h SD[4] (ending at ZT 4), wake (ZT16)	~20% increase in postsynaptic GluA1, pSer845-GluA, and PKA at ZT16 relative to ZT4, no changes with SD	mice	8-10 weeks	forebrain (synaptosomes)	[25]
Anatomy:					
2 h sleep vs. SD[5]	spine/filopodia formation equal between sleep and SD, elimination increased ~10% across sleep relative to SD	mouse	3 weeks	somatosensory cortex, layer 5 pyramidal neurons	[26]
sleep (ZT6), 6 h SD (ending at ZT 6), wake (ZT18)[6]	during wake and SD, axon spine interface size increased ~10-15% (only affected smaller spines; largest unaffected)	mouse	4 weeks	primary motor and somatosensory cortex	[15]
Electrophysiology:					
ZT1 vs ZT5-6, 4 h SD (ending at ZT4)[7]	decreased firing rates in fast-spiking interneurons at ZT5-6 vs. ZT0, increased multiunit firing after SD	rat	13-16 weeks	barrel cortex and frontal cortex	[14]
4 h sleep vs. SD[1]	increased mEPSC amplitudes and frequencies after SD	mouse/rat	3-4 weeks/4-8 weeks	frontal cortex	[33]
spontaneous sleep and wake bouts	firing rates increase across wake and decrease across sleep; ratio of interneuron-to-pyramidal neuron firing higher during wake than sleep	rat	unknown (adult)	hippocampal area CA1	[30]

<div align="center">Evidence for synaptic strengthening during sleep:</div>

Manipulation	*Key findings*	*Species*	*Age*	*Brain area*	*Reference*
Biochemistry:					
1 h sleep vs. SD[8] following monocular visual experience	increased synaptic BDNF protein levels during sleep (but not	cat	postnatal day P28-40	primary visual cortex (homogenate and synaptoneurosomes)	[134]

	SD); decreased Arc protein levels after SD				
1 or 2 h sleep vs. SD[8] following monocular visual experience	5-10 fold increase in pCaMKIIa, pERK, and pSer831-GluA1 during sleep (but not SD)	cat	postnatal day P28-40	primary visual cortex (homogenate)	[10]
1, 3, or 6 h of *ad lib* sleep following two-way active avoidance or sham training	post-training increases in pCREB, BDNF and Arc protein proportional to post-training increases in REM PGO wave density	rat	unknown (adult)	hippocampus, amygdala, frontal and occipital cortex (homogenate)	[143]
Anatomy:					
5 h sleep vs. SD[8]	~20% decrease in spine density after SD	mouse	2-3 months	hippocampal area CA1 pyramidal neurons	[7]
~7 h sleep vs. SD[1] following motor learning	~50% decrease in spine formation across period of SD relative to sleep	mouse	unknown (adult)	M1 layer 5 pyramidal neurons	[6]
Electrophysiology:					
spontaneous sleep and wake bouts	increased amplitude evoked field potential responses following NREM sleep	cat	unknown (adult)	somatosensory cortex	[138]
3 or 5 h sleep vs. SD[9]	disruption of PKA-dependent forms of LTP after SD	mouse	2-4 months	hippocampal area CA1	[117,121]
6 h sleep vs. SD[9] following novel visual experience	selective firing rate responses increased after sleep (but not SD); neuronal firing rates increase across bouts of NREM and REM (but not wake)	mouse	1-4 months	primary visual cortex	[8]

[1] SD via tactile stimulation
[2] SD via air puffs, exposure to novel objects
[3] SD via exposure to novel objects
[4] SD via cage change
[5] SD via exposure to novel objects and gentle touch
[6] during both SD and wake phase (not sleep phase) mice were given access to a running wheel and exposed to novel objects
[7] SD via exposure to novel objects and acoustic stimuli
[8] SD via novel objects, acoustic stimuli, tactile stimulation, and floor rotation
[9] SD via cage tapping, shaking, and nest disturbance

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CHAPTER II

Sleep Loss Drives Brain Region- and Cell Type-Specific Alterations in Ribosome-Associated Transcripts Involved in Synaptic Plasticity and Cellular Timekeeping

2.1 Abstract

Sleep and sleep loss are thought to impact synaptic plasticity, and recent studies have shown that sleep and sleep deprivation (SD) differentially affect gene transcription and protein translation in the mammalian forebrain. However, much less is known regarding how sleep and SD affect these processes in different microcircuit elements within the hippocampus and neocortex - for example, in inhibitory vs. excitatory neurons. Here we use translating ribosome affinity purification (TRAP) and in situ hybridization to characterize the effects of sleep vs. SD on abundance of ribosome-associated transcripts in Camk2a-expressing (Camk2a+) pyramidal neurons and parvalbumin-expressing (PV+) interneurons in mouse hippocampus and neocortex. We find that while both Camk2a+ neurons and PV+ interneurons in neocortex show concurrent SD-driven increases in ribosome-associated transcripts for activity-regulated effectors of plasticity and transcriptional regulation, these transcripts are minimally affected by SD in hippocampus. Similarly we find that while SD alters several ribosome-

associated transcripts involved in cellular timekeeping in neocortical Camk2a+ and PV+ neurons, effects on circadian clock transcripts in hippocampus are minimal, and restricted to Camk2a+ neurons. Taken together, our results indicate that SD effects on transcripts destined for translation are both cell type- and brain region-specific, and that these effects are substantially more pronounced in the neocortex than the hippocampus. We conclude that SD-drivenalterations in the strength of synapses, excitatory-inhibitory balance, and cellular timekeeping are likely more heterogeneous than previously appreciated. ;;

2.2 Introduction

Sleep is essential for optimal brain function, but the underlying biological mechanisms are largely unknown. Prior work aimed at addressing this question has used molecular profiling of mRNA and protein abundance, in a number of brain areas, to characterize changes caused by experimental SD (Cirelli et al., 2004; Mackiewicz et al., 2007; Noya et al., 2019; Poirrier et al., 2008; Vecsey et al., 2012). Transcriptomic changes reported after SD in the brain have led to specific hypotheses regarding the biological underpinnings of cognitive disruptions associated with sleep loss (Belenky et al., 2003; Dinges et al., 1997; Mednick et al., 2003; Stickgold, 2005). For example, the synaptic homeostasis hypothesis (Tononi and Cirelli, 2006) proposes that synapses throughout the brain are strengthened during periods of wake and weakened during periods of sleep. The proposal of this hypothesis was initially based on results from transcriptomic studies in mice, showing higher expression of both immediate early genes (IEGs) and several other genes involved in synaptic plasticity after periods of SD vs. sleep (Cirelli et al., 2004; Cirelli et al., 1996; Cirelli and Tononi, 2000).

However, there may be more heterogeneity in responses to SD across the brain than previously thought. For example, SD-driven transcript changes may vary between different brain structures (Mackiewicz et al., 2007; Terao et al., 2006; Vecsey et al., 2012). We have recently shown that while SD increases expression of the plasticity-mediating IEG *Arc* and Arc protein abundance in neocortical areas (e.g., primary somatosensory cortex; S1), it simultaneously decreases *de novo* synthesis of Arc in the hippocampal dentate gyrus (DG). Indeed, recent data have suggested that SD could differentially impact neuronal activity and dendritic spine density in hippocampal vs. neocortical structures (de Vivo et al., 2017; Havekes and Aton, 2020; Havekes et al., 2016; McDermott et al., 2003; Ognjanovski et al., 2018; Raven et al., 2019; Vyazovskiy et al., 2009). Because cognitive processes reliant on the hippocampus, such as episodic memory consolidation (Havekes and Abel, 2017; Saletin and Walker, 2012), seem particularly susceptible to disruption by SD, a critical unanswered question is whether SD differentially impacts network activity and plasticity in the two structures. Beyond this, within brain structures, there may be heterogeneity in the responses of different neuronal subtypes to SD. For example, within the neocortex, fast-spiking interneurons, or neurons with greater firing rates, appear to have differential firing rate changes across periods of sleep (Clawson et al., 2018; Vyazovskiy et al., 2009). Consistent with this idea, synaptic excitatory-inhibitory (E-I) balance was recently shown to vary in neocortex over the course of the day in a sleep-dependent manner (Bridi et al., 2020). Moreover, while most neocortical neurons fire at lower rates during slow wave sleep (SWS) vs. wake, some

subclasses of neocortical neurons are selectively sleep-active (Gerashchenko et al., 2008).

Here we aimed to better characterize brain region- and cell type-specific changes evoked in the nervous system during SD. We used cell type-specific translating ribosome affinity purification (TRAP) (Sanz et al., 2019) to profile SD-mediated changes in ribosome-associated mRNAs in two prominent hippocampal and neocortical cell types – Camk2a+ pyramidal neurons and PV+ interneurons. Because interactions between these two cell types are critical for mediating state-dependent sensory plasticity and memory consolidation (Aton et al., 2013; Kuhlman et al., 2013; Ognjanovski et al., 2018; Ognjanovski et al., 2017), we characterized SD-driven changes in ribosome-associated transcripts encoding transcription-regulating IEGs, plasticity effector proteins, and circadian clock components in these two cell types. We find that SD generally causes more modest changes to these transcripts in hippocampal vs. neocortical circuits, and in PV+ interneurons vs. Camk2a+ neurons. Together our data suggest that the effects of SD on the brain are more heterogeneous than previously thought, and indicate region- and cell type-dependent differences in SD's impact which may have important implications for brain function.

2.3 Materials and Methods

Mouse handling and husbandry

All animal procedures were approved by the University of Michigan Institutional Animal Care and Use Committee (PHS Animal Welfare Assurance number D16-00072

[A3114-01]). Animals were maintained on a 12:12h light/dark cycle (lights on at 8AM) with food and water provided *ad lib*. Mice expressing Cre recombinase in Camk2a+ neurons or PV+ interneurons (B6.Cg-Tg(Camk2a-cre)T29-1Stl/J or B6;129P2-*Pvalb*$^{tm1(cre)Arbr}$/J; Jackson) were crossed to RiboTag mice (B6N.129-Rpl22$^{tm1.1Psam}$/J; Jackson) to express HA-tagged Rpl22 protein in these neuron populations. 3-5 month old mice were individually housed one week prior to all experiments (with beneficial enrichment), and were habituated to handling for five days prior to experiments. Following habituation, and beginning at lights on (ZT0), mice were either allowed *ad lib* sleep in their home cage or were sleep deprived by gentle handling (Delorme et al., 2019; Durkin and Aton, 2016; Durkin et al., 2017). For sleeping animals, sleep behavior was visually scored at 5-min or 2-min intervals (for 6-h and 3-h SD, respectively), based on immobility and assumption of characteristic sleep postures. Previous research from our lab has shown that wake time over the final 45 min of the experiment correlates with *Arc* IEG expression in neocortex (Delorme et al., 2019). Thus to reduce the probability of confounding results from freely-sleeping mice, mice in the Sleep groups that spent > 60% of the final 45 min of the experiment in wake were excluded from subsequent analysis. All mice were sacrificed with an overdose of pentobarbital (Euthasol) prior to tissue harvest.

Translating Ribosome Affinity Purification (TRAP)

TRAP was performed using methods described in prior studies (Sanz et al., 2009), with minor modifications. Following 3-6 h of *ad lib* sleep or SD, animals were euthanized with an overdose of pentobarbitol (Euthasol), their brains extracted, and hippocampi/cortices dissected in dissection buffer (1x HBSS, 2.5 mM HEPES [pH 7.4], 4

mM NaHCO$_3$, 35 mM glucose, 100 μg/ml cycloheximide). Tissue was then transferred to glass dounce column containing 1 mL of homogenization buffer (10 mM HEPES [pH 7.4], 150 mM KCl, 10 mM MgCl$_2$, 2 mM DTT, cOmplete™ Protease Inhibitor Cocktail [Sigma-Aldrich, 11836170001], 100 U/mL RNasin® Ribonuclease Inhibitors [Promega, N2111], and 100 μg/mL cycloheximide) and manually homogenized on ice. Homogenate was transferred to a 1.5 mL LoBind tubes (Eppendorf) and centrifuged at 1000×g at 4°C for 10 min. Supernatant was then transferred to a new tube, 90 μL of 10% NP40 was added, and samples were allowed to incubate for 5 min. Following this step, the supernatant was centrifuged at maximum speed for 10 min at 4°C, transferred to a new tube, and mixed with 10 μl of HA-antibody (Abcam, ab9110) (Jiang et al., 2015; Shigeoka et al., 2018). Antibody binding proceeded by incubating the homogenate-antibody solution for 1.5 hours at 4°C with constant rotation. During the antibody rinse, 200 μl of Protein G Dynabeads (ThermoFisher, 10009D) were washed 3 times in 0.15 M KCl IP buffer (10mM HEPES [pH 7.4], 150 mM KCl, 10 mM MgCl$_2$, 1% NP-40) and incubated in supplemented homogenization buffer (10% NP-40). Following this step, supplemented buffer was removed, the homogenate-antibody solution was added directly to the Dynabeads, and the solution incubated for 1 h at 4°C with constant rotation. After incubation, the RNA-bound beads were washed four times in 900 μL of 0.35 M KCl (10 mM HEPES [pH 7.4], 350 mM KCl, 10 mM MgCl$_2$, 1% NP40, 2 mM DTT, 100 U/mL RNasin® Ribonuclease Inhibitors [Promega, N2111], and 100 μg/mL cycloheximide). During the final wash, beads were placed onto the magnet and moved to room temperature. After removing the supernatant, RNA was eluted by vortexing the beads vigorously in 350 μL RLT (Qiagen, 79216). Eluted RNA was purified using RNeasy Micro kit (Qiagen).

Quantitative real-time PCR (qPCR) and stability analysis

Quantitative real-time PCR (qPCR) experiments were performed as described previously (Delorme et al., 2019). Briefly, purified mRNA samples were quantified by spectrophotometry (Nanodrop Lite; ThermoFisher) and diluted to equal concentrations. 20-500 ng of mRNA was used to synthesize cDNA using iScript's cDNA Synthesis Kit (Bio-Rad), cDNA diluted 1:10 in RNAse-free H_2O, and measured using a CFX96 Real-Time System. Primers were designed for these studies, with the exception of Homer1a, for which sequences were established in a prior study (Mikhail et al., 2017). Primer specificity was confirmed using NIH Primer Blast (see **Table 2.4** for primer sequences). Three technical replicates were used for each sample. Relative changes in gene expression between sleep and SD were quantified using the ΔΔCT method, and these fold changes are presented on a log scale (log_2 transformed value equivalent to ΔΔCT) with propagated errors. All statistical analyses were performed on ΔCT values.

Reference (housekeeping) genes for normalization were chosen for each experiment based on three measures: intragroup variability, intergroup variability, and an overall stability measure derived from total variance. Special emphasis was placed on selecting pairs of reference transcripts with countervailing intergroup differences. These measures were calculated using Normfinder (Andersen et al., 2004) and RefFinder (Xie et al., 2012) software. Because Normfinder is better suited for large sample sizes, RefFinder was used to validate Normfinder rankings and ensure genes with low (or opposite-direction) intergroup variability were chosen as housekeeping pairs. Stability measures were calculated for each sleeping condition, region, and mRNA population and

repeated for mRNAs purified from *PV::RiboTag* and *Camk2a::Ribotag* mice (**Table 2.1**). The arithmetic mean of each housekeeping pair was then used to normalize target gene expression. As a final measure of housekeeping stability, we calculated each pairs' fold change between mice in SD and Sleep groups.

RNAScope *in situ* hybridization

Fluorescent *in situ* hybridization was performed on 14-□m coronal sections taken from fixed-frozen brains of Sleep ($n = 6$) and SD ($n = 6$) mice. Section coordinates (1–3.0 mm lateral, −1.4 to −2.8 mm posterior to Bregma) were similarly distributed between Sleep and SD conditions (**Figure S2C**).The RNAScope Multiplex Fluorescent Reagent Kit v2 with 4-plex ancillary kit was used to label *Arc, Homer1a, Cfos,* and *Pvalb* transcripts (**Figure S2**). Prior to probe incubation, slices were pretreated with hydrogen peroxide (10 min, room temperature), Target Retrieval Reagent (5 min 99°C), and RNscope Protease III (30 min, 40°C). Slices were incubated with custom-synthesized *Arc* (20 bp, Target Region: 23-1066, 316911-C3, Advanced Cell Diagnostics), *Cfos* (20 bp, Target Region: 407-1427, 316921-C1, Advanced Cell Diagnostics), *Homer1a* (6 bp, Target Region: 1301-1887m 433941-C2, Advanced Cell Diagnostics), and *Pvalb* 16 (16 bp, Target RegionL 2-885, 421931-C4, Advanced Cell Diagnostics). Probes were chosen so as to overlap with regions amplified by qPCR primer pairs. *Arc, Cfos, Homer1a, and Pvalb* were hybridized to Opal Dyes 620 (FP1495001KT, Akoya Biosciences), 570 (FP1488001KT, Akoya Biosciences), 690 (FP1497001KT, Akoya Biosciences), and 520 (FP1487001, Akoya Biosciences), respectively, for visualization. Positive and negative control probes were used in parallel experiments to confirm specificity of hybridization.

Immunohistochemistry

For immunohistochemical validation of appropriately cell targeted HA expression in RiboTag-expressing mice, *Camk2a::RiboTag* and *PV::RiboTag* mice from Sleep (*n* = 6) and SD (*n* = 6) groups were sacrificed and perfused with PBS followed by 4% paraformaldehyde. 50-μm brain sections were blocked with normal goat serum for 2 h and incubated overnight using biotin-conjugated anti-HA (Biolegend 901505, 1:500) and anti-parvalbumin (Synaptic Systems 195 004, 1:500) antibodies at 4°C. The following day, sections were stained with Streptavidin-Alexa Fluor® 647 (Biolegend 405237) and Alexa Fluor® 555 Goat Anti-Guinea pig IgG H&L (Abcam ab150186). Stained sections were coverslipped in ProLong Gold Antifade Reagent (ThermoFisher, P36930). Fluorescence intensity was used to identify HA -expressing (HA$^+$) cells, PV-expressing (PV$^+$) cells, and overlapping cells within the DG, CA1, CA3, and neocortex. To account for differences in localization and spread of antibody staining, both PV$^+$ HA-expressing cells and HA$^+$ PV-expressing cells were identified, and overlap was quantified in terms of both cell count and cell area (**Figure 2.1**). Quantification was performed using the semi-automated protocol detailed below.

Imaging and quantification

RNAScope probe fluorescence signals were captured and stitched using a 40× objective lens on a Leica 3D STED SP8. Immunostained brain 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). Two images per region (one per

hemisphere) were quantified for each animal. Total fluorescence dot number and average intensity of a single dot calculated as described here (ACDBio, 2017), for PV+ and non-PV+ regions of interest (ROIs) within granule (dentate gyrus), pyramidal (CA1, CA3), and cortical layers 1-6 (layers were manually isolated using a freehand tool by a scorer blind to experimental condition). Fluorescence intensity and expression overlap were calculated using a semi-automated protocol curated by blinded scorer. 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. Identified areas were labeled as IEG or PV and manually edited to refine labeling, select for representative dots, and remove artifacts (manual editing was not used to label any additional IEG expression). Finalized labeling was used to delineate PV+ and non-PV+ ROIs, select for background area (area in the ROI minus areas of labeled expression), and identify IEG+ PV+ cells (**Figure S3**). Intensity values from ROIs, background, and selected dots were used to calculate fluorescence dots/area. Average background intensity was calculated as the fluorescence intensity of the selected background area per unit area. The average intensity of a single fluorescent dot was calculated for each transcript as the intensity of manually selected representative dots within the ROI, minus the average background intensity multiplied by the area, divided by the total number of selected dots. Dot intensity values did not differ between Sleep and SD mice for specific transcripts. The total fluorescent dot number within each ROI was calculated by subtracting average background intensity from total ROI fluorescence intensity, multiplied by total area, divided by average dot intensity.

2.4 Results: TRAP-based characterization of ribosome-associated transcripts in Camk2a+ and PV+ neuronal populations

To quantify how ribosome-associated transcripts in different neuronal populations within the hippocampus and neocortex are affected by sleep loss, we crossed RiboTag transgenic mice (with Cre recombinase-dependent expression of HA-tagged Rpl22 protein) to either Camk2a-Cre or PV-Cre transgenic lines (Sanz et al., 2019) (**Figure 2.1A**). Appropriate cell type-specific expression of Rpl22[HA] in *Camk2a::RiboTag* and *PV::RiboTag* mice was verified using immunohistochemistry (**Figure 2.1B-F**). HA expression was largely circumscribed to the intended cell type. For example, 86.7 ± 1.5% and 79.4 ± 1.8% of HA+ neurons within the hippocampus and neocortex of *PV::RiboTag* mice co-expressed PV peptide. In both *Camk2a::RiboTag* and *PV:RiboTag* mice, expression of HA in non-targeted cell types of the hippocampus (based on lack of co-immunostaining for Camk2a or PV) was minimal (3.6 ± 0.2% and 13.3 ± 1.5%, respectively; **Figure 2.1D-F**).

We next validated cell type-specificity of ribosome-associated transcripts isolated from transgenic mouse lines. Following a period of *ad lib* sleep of sleep deprivation (SD) starting at lights on (ZT0), hippocampi and neocortex were dissected, and ribosome-associated mRNAs were isolated (Sanz et al., 2019). We compared abundance of cell type-specific transcripts between RiboTag affinity purified mRNA and Input mRNA from whole hippocampus or neocortex homogenate using qPCR. Enrichment or de-enrichment of these cell markers was compared with a null hypothetical value of 0 using one-sample t-tests. We found that ribosomal-associated transcripts from both the neocortex and

hippocampus of *Camk2a::RiboTag* mice de-enriched for markers of glial cell types (*Mbp, Gfap*), non-PV+ inhibitory neurons (*Npy, Sst*), PV interneurons (*Gad67, Pvalb*), and *Vglut1* relative to Input (**Figure 2.1G-H**). Hippocampal enrichment patterns mirrored those of the neocortex with the exception of *Vglut2*, which was significantly enriched relative to Input. Ribosome-associated transcripts from *PV::RiboTag* mice de-enriched for markers of glial (*Mbp, Gfap*), non-PV+ inhibitory (*Npy, Sst*), and excitatory neurons (*Vglut1, Vglut2, Camk2a*) while enriching for PV+ interneuron markers (*Pvalb, Gad67*) relative to Input. We made comparisons of cell type-specific transcript enrichment separately for mice which were either allowed *ad lib* sleep or sleep deprived (SD) over the first 3 or 6 h after lights on (i.e., from ZT0-3, or ZT0-6; **Figure S1A**). No substantial differences in enrichment patterns were observed between Sleep and SD mice (*N.S.*, Holm-Sidak *post hoc* test). These data confirm the high degree of specificity of TRAP-based profiling for ribosomal transcripts from Camk2a+ principal neurons and PV+ interneurons.

2.5 Results: SD-driven changes in ribosome-associated plasticity-related mRNAs vary with cell type and brain structure

We first quantified a subset of transcripts encoding for proteins involved in synaptic plasticity (i.e., plasticity effectors) whose expression levels have been reported previously as altered by SD - *Arc, Homer1a, Narp, and Bdnf* (Cirelli et al., 2004; Maret et al., 2008). Ribosome-associated transcript abundance was first quantified in Camk2a+ neocortical and hippocampal neuron populations after 3 h of *ad lib* sleep (Sleep; $n = 4$) or SD ($n = 5$), starting at lights on (ZT0). Consistent with previous findings (Cirelli et al., 2004), 3-h SD significantly increased neocortical *Arc* ($p < 0.001$, Holm–Sidak *post hoc* test) and
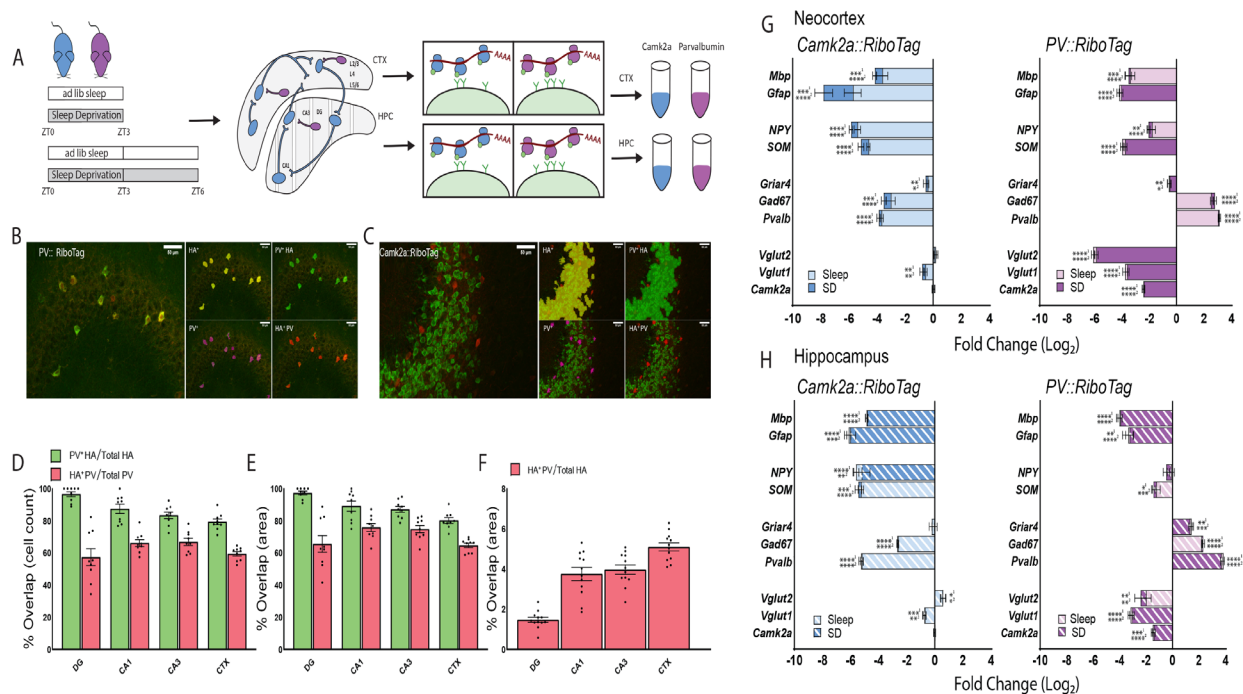
Figure 2.1. Experimental design and validation of TRAP. (A) *Camk2a::RiboTag* (blue) and *PV::RiboTag* (magenta) transgenic mice were sacrificed after a 3- or 6-h period of *ad lib* sleep (Sleep) or sleep deprivation (SD) starting at lights on (ZT0). Ribosome-associated mRNAs were affinity purified from hippocampus and neocortex. (**B**) HA expression in PV+ interneurons was validated with immunohistochemistry in *PV::RiboTag* mice, with automated detection of HA (green fluorescence, labeled in yellow) and PV (red fluorescence, labeled in purple) expression. Areas of overlapping fluorescence were then identified; to account for differences in antibody staining, both PV$^+$ HA-expressing areas (green) and HA$^+$ PV-expressing areas (red) were identified. Scale bars = 50 μm. (**C**) Example of automated protocol used in *Camk2a::RiboTag* mice to quantify non-specific expression. (**D-E**) HA expression presented as proportion of overlapping cells vs. total cell count (**D**) and total area (**E**) in *PV::RiboTag* sections. (**F**) PV$^+$ HA-expressing areas over total HA$^+$ area in *Camk2a::RiboTag* sections. (**G**) Enrichment of markers for glia (*Mbp, Gfap*), non-PV+ inhibitory neurons (*NPY, SOM*), PV+ neurons (*Griar4, Gad67, PV*), and excitatory neurons (*Vglut1, Vglut2, Camk2a*) calculated as ΔΔCT between affinity purified (RiboTag) mRNA and Input mRNA from neocortex. Data presented as log(2) transformed fold changes. (**H**) Enrichment values for *Camk2a::RiboTag* and *PV::RiboTag* hippocampi. Gene expression was normalized to housekeeping gene pairs according to their respective condition (see **Table 1**). Values indicate mean ± SEM with propagated error; *, **, ***, and **** indicate $p < 0.05$, $p < 0.01$, $p < 0.001$, and $p < 0.0001$, respectively, one sample t-test against hypothetical value of 0.

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Homer1a (*p* < 0.01) (Maret et al., 2008) ribosome-associated mRNA (**Figure 2.2A**). In contrast, and consistent with recent data (Delorme et al., 2019), 3-h SD significantly increased *Homer1a* abundance on hippocampal ribosomes (*p* < 0.01), but did not significantly affect *Arc* abundance (*N.S.*, Holm–Sidak *post hoc* test). Overall patterns of transcript abundance for the plasticity-regulating proteins *Bdnf* and *Narp* followed a similar trend, with unchanged levels in hippocampal Camk2a+ neurons (*N.S*, Holm–Sidak *post hoc* test), and modestly (but not significantly) increased levels in neocortical neurons (*Narp* and *Bdnf*, *N.S.*). After more prolonged (6-h) SD (*n* = 6 mice/group), ribosome-associated *Arc* (*p* < 0.0001), *Homer1a* (*p* < 0.0001), and *Bdnf* (*p* < 0.01) transcripts were all increased in neocortical Camk2a+ neurons, whereas *Arc* (*p* < 0.01) and *Homer1a* (*p* < 0.0001) were increased in hippocampal Camk2a+ neurons (**Figure 2.2B**).

We next quantified ribosome-associated transcript abundance in PV+ interneuron populations from the neocortex (*n* = 4 mice/group) and hippocampus (*n* = 4 and *n* = 5 mice for Sleep and SD). 3-h SD significantly increased *Arc* (*p* < 0.001, Holm–Sidak *post hoc* test) abundance in neocortical PV+ interneurons, but had no effect on transcript abundance for plasticity-related proteins in hippocampal PV+ interneurons (*N.S.*, Holm–Sidak *post hoc* test). 6-h SD increased abundance of these transcripts in the neocortical PV+ interneuron population (*n* = 5 and *n* = 6 mice for Sleep and SD) in a manner similar to the Camk2a+ neuronal population (*Arc, p* < 0.0001; *Homer1a, p* < 0.0001; *Narp, p* < 0.05; *Bdnf, p* < 0.01). In contrast, 6-h SD caused no significant change in any of the ribosome-associated transcripts' abundance in hippocampal PV+ interneurons (*n* = 6 mice/group).
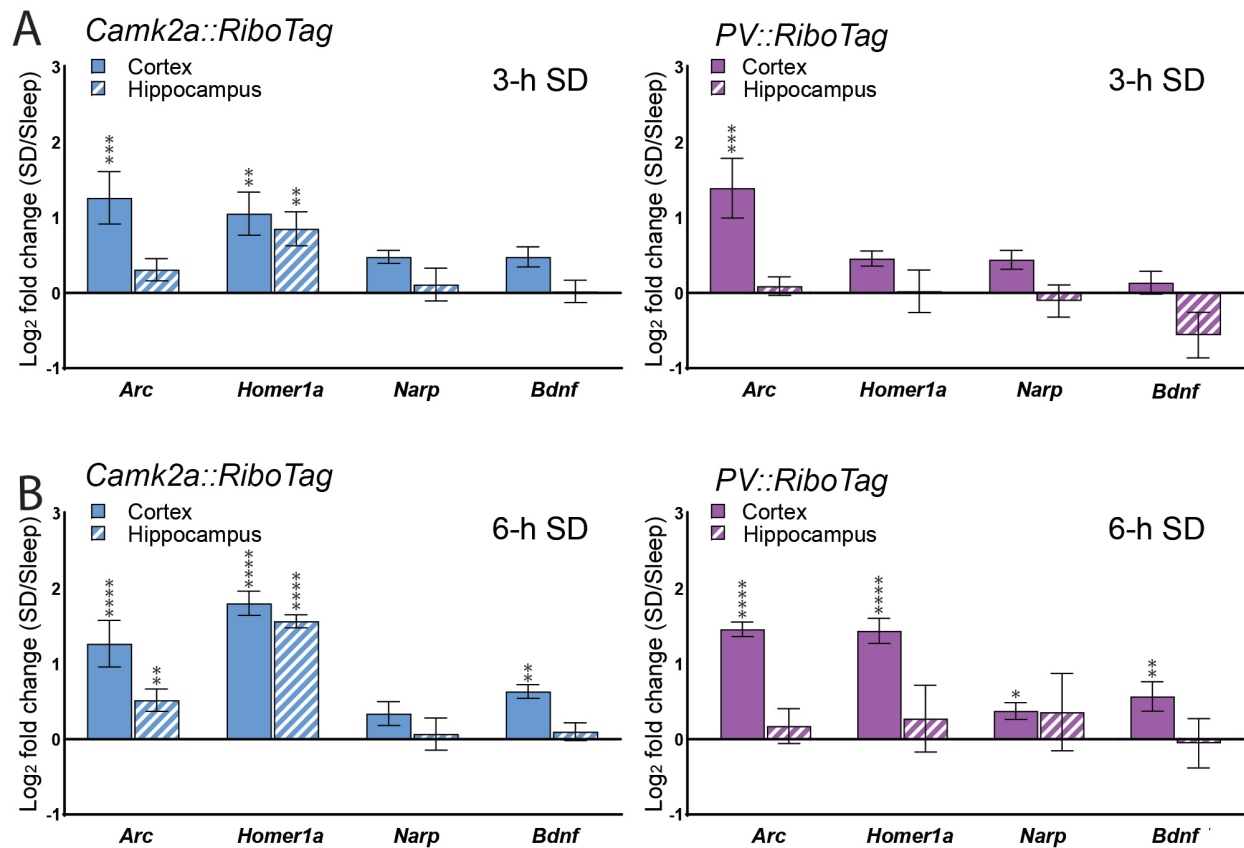
Figure 2.2. SD increases ribosome-associated plasticity effector transcripts in a cell type- and region-specific manner. (A) 3-h SD significantly increased *Arc* and Homer1alevels on ribosomes from Camk2a+ neocortical (solid) neurons; only *Homer1a* increased in hippocampal (dashed) neurons. 3-h SD significantly increased *Arc* on ribosomes from PV+ interneurons in neocortex; no significant change was observed in the hippocampal PV+ interneuron population. **(B)** *Arc*, *Homer1a*, and *Bdnf* significantly increased after 6-h SD in Camk2a+ neocortical neurons; *Arc* and *Homer1a* were increased within the Camk2a+ hippocampal population. All effector transcript levels were significantly elevated after 6-h SD in PV+ interneurons in neocortex; no significant change was observed in the hippocampal PV+ population. Transcript level changes are presented as a log₂ fold change between SD and *ad lib* sleep mice. All statistical tests were done on ΔCT values. Values indicate mean ± SEM with propagated error from *ad lib* sleep and SD groups; *, **, ***, and **** indicate $p < 0.05$, $p < 0.01$, $p < 0.001$, and $p < 0.0001$, respectively, Holm–Sidak *post hoc* test vs. Sleep.

2.6 Results: SD differentially affects abundance of ribosome-associated mRNAs encoding activity-dependent transcription regulators based on cell type in hippocampus vs. neocortex

To better characterize how SD affects activity-regulated pathways in Camk2a+ and PV+ populations, we quantified ribosome-associated transcript abundance for IEGs encoding transcription regulatory factors - *Npas4*, *Cfos*, and *Fosb*. We first quantified transcript abundance in Camk2a+ neocortical and hippocampal neuronal populations after 3-h of *ad lib* sleep (Sleep; *n* = 4) or SD (*n* = 5), starting at lights on (ZT0). 3-h SD produced no significant change in ribosome-associated transcript abundance in Camk2a+ neocortical cells (*N.S.* for all transcripts, Holm–Sidak *post hoc* test) while significantly increasing *Cfos* abundance in the hippocampus ($p < 0.05$; **Figure 2.3A**). After prolonged (6-h) SD (*n* = 6 mice/group; **Figure 2.3B**), neocortical *Npas4* ($p < 0.01$), *Cfos* ($p < 0.0001$) and *Fosb* ($p < 0.01$) abundance increased on ribosomes in Camk2a+ neurons. In the hippocampus, ribosome-associated *Npas4* ($p < 0.001$), *Cfos* ($p < 0.0001$), and *Fosb* ($p < 0.0001$) all increased in abundance in Camk2a+ neurons after 6-h SD.

2.7 Results: Subregion- and layer-specific effects of SD on mRNA abundance in PV+ and non-PV+ neurons

Recent findings suggest that effects of SD on transcription and translation may be more region- and subregion-specific than previously thought (Delorme et al., 2019; Havekes and Aton, 2020). To more precisely characterize region-specific changes in mRNA abundance after SD, we used fluorescence *in situ* hybridization to visualize *Pvalb*,
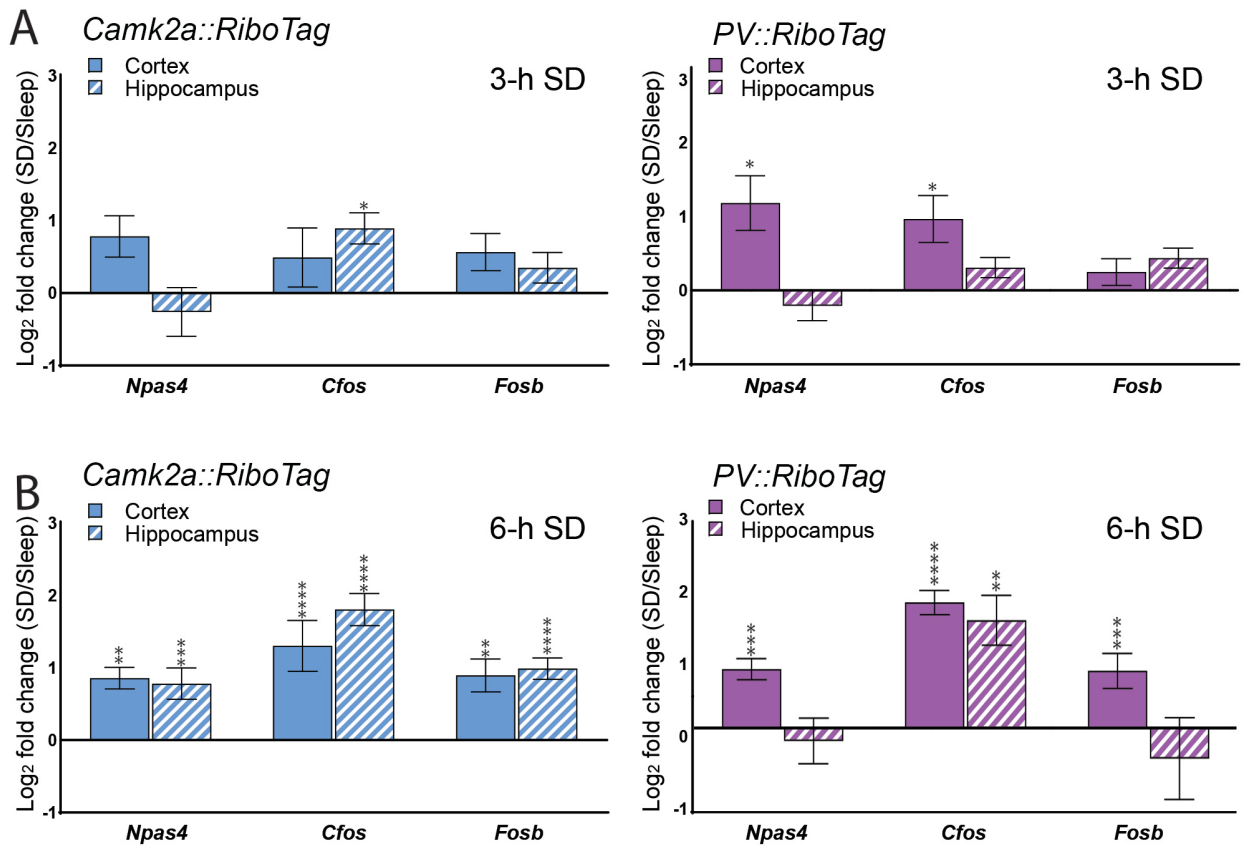
Figure 2.3. SD increases ribosome-associated transcripts encoding immediate-early transcription regulators in a cell type- and region-specific manner. (A) 3-h SD had no significant effect on IEG transcript levels on ribosomes from Camk2a+ neocortical (solid) neurons; only *Cfos* increased in hippocampal (dashed) neurons. 3-h SD significantly increased *Npas4* and *Cfos* on ribosomes from PV+ interneurons in neocortex, but did not affect IEG abundance on ribosomes from hippocampal PV+ neurons. **(B)** 6-h SD significantly increased *Npas4, Cfos*, and *Fosb* levels in Camk2a+ neocortical neurons, Camk2a+ hippocampal neurons, and PV+ neocortical interneurons. Only *Cfos* significantly increased in the PV+ hippocampal population with 6-h SD. Transcript level changes are presented as a $\log_2$ fold change between SD and *ad lib* sleep mice. All statistical tests were done on ΔCT values. Values indicate mean ± SEM with propagated error from *ad lib* sleep and SD groups; *, **, ***, and **** indicate $p < 0.05$, $p < 0.01$, $p < 0.001$, and $p < 0.0001$, respectively, Holm–Sidak *post hoc* test vs. Sleep.

Arc, *Homer1a*, and *Cfos* transcripts in C57Bl6/J mice after 6-h SD (n = 6) or *ad lib* sleep (n = 5)(**Figure 2.4A, Figure 2.5A-B, Figure S1B, Figure S2**). Transcripts were quantified separately in neocortical layers 1-6 and DG, CA3, and CA1 hippocampal subregions. *Pvalb* expression was used to discriminate expression in PV+ interneurons from that in non-PV+ (mainly pyramidal) neurons. Regions of interest (ROIs) for PV+ interneurons and non-PV+ regions were identified separately and total transcript expression (total fluorescence dot number) was normalized to the area of their respective ROI. We first quantified mRNA abundance after Sleep vs. SD among non-PV+ cells in neocortical regions overlying dorsal hippocampus (including S1)(**Figure 2.4B**). Across neocortex as a whole, SD significantly increased *Arc* in non-PV+ neurons (Sleep = 24.8 ± 10.3 vs. SD = 79.2 ± 10.1 dots/mm^2, p < 0.05, Holm–Sidak *post hoc* test), and showed a tendency for increasing *Cfos* (Sleep = 8.6 ± 3.9 vs. SD = 26.2 ± 5.1 dots/mm^2, p = 0.053) and *Homer1a* (Sleep = 1.4 ± 0.5 vs. SD = 7.8 ± 2.6 dots/mm^2, p = 0.056). Expression was also quantified in individual neocortical layers. The largest effects of SD were seen for *Homer1a* and *Cfos* in layers 4 (*Homer1a*: Sleep = 1.6 ± 0.6 vs. SD = 7.8 ± 2.2 dots/mm^2, *Cfos*: Sleep = 13.5 ± 6.4 vs. SD = 40.5 ± 7.1 dots/mm^2) and 5 (*Homer1a*: Sleep= 1.5 ± 0.4 vs SD=9.5 ± 2.8 dots/mm^2, *Cfos*: Sleep = 8.8 ± 3.8 vs. SD = 34.5 ± 6.9 dots/mm^2, p < 0.05). SD increased *Arc* dots/mm^2 significantly across layers 2/3 (Sleep = 15.2 ± 5.8 vs. SD = 45.8 ± 3.7 dots/mm^2, p < 0.01, unpaired *t*-test), 4 (Sleep = 36.3 ± 14.3 vs. SD=137.5 ± 17.7 dots/mm^2, p < 0.01), and 5 (Sleep = 21.7 ± 8.2 vs. SD = 81.7 ± 12.8 dots/mm^2, p < 0.05) (**Figure 2.4B**). No changes in expression were observed with SD in layer 6, and layer 1 expression was not analyzed due to low overall expression and cell density. In dramatic contrast to the relatively large changes in non-PV+ transcript abundance with
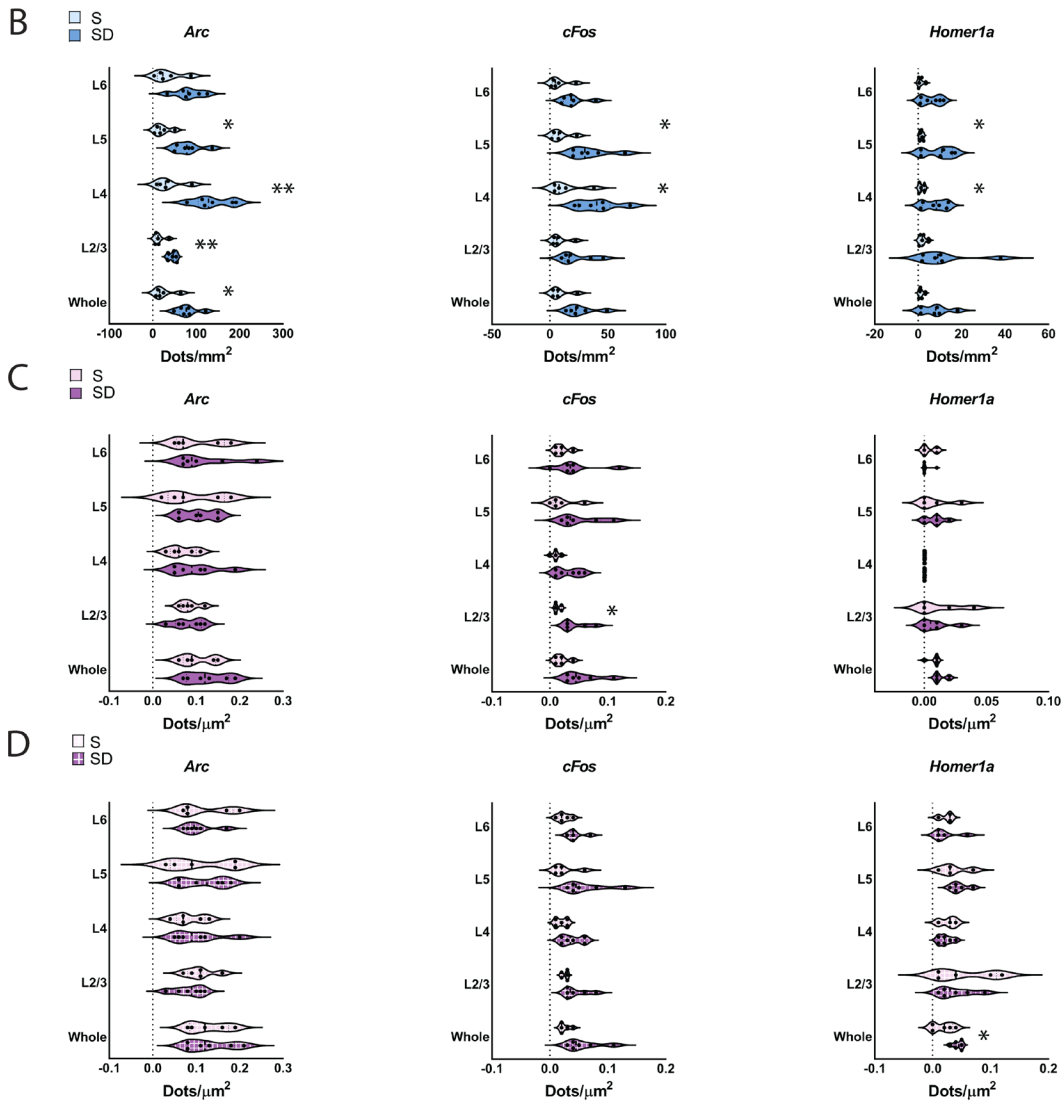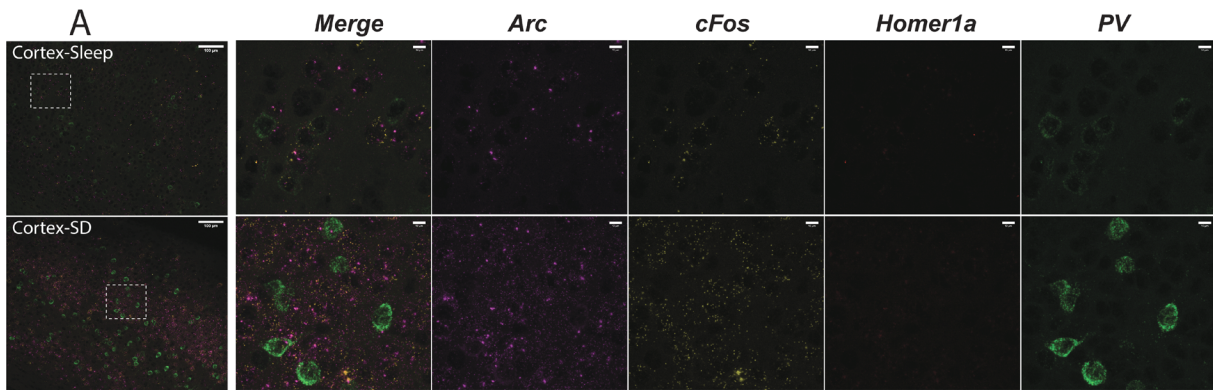
Figure 2.4. Layer-specific induction of IEG expression increases in neocortex after SD. (A) Representative images show neocortical IEG expression after 6 h of *ad lib* sleep (*n* = 5 mice) or SD (*n* = 6 mice). Inset regions are shown at higher magnification on right. Scale bars indicate 100 μm and 10 μm respectively. **(B)** 6-h SD significantly increased *Arc* dots/mm² among non-PV+ cells in whole cortex and layers 2/3, 4 and 5, and *Cfos* and *Homer1a* dots/mm² in layers 4 and 5. **(C)** 6-h SD significantly increased *Cfos* dots/μm² among *Pvalb*+ cells (magenta) in layer 2/3; no other significant changes were observed. **(D)** When analysis was restricted to IEG+ *Pvalb*+ cells (magenta, box pattern), SD significantly increased *Homer1a* dots/μm² among *Homer1a*+ *Pvalb*+ cells in whole cortex; no other significant changes were observed. Analysis circumscribed to non-PV ROI (without *Pvalb* expression) and PV ROI (with *Pvalb* expression). Violin plots show distribution of values for individual mice; * and ** indicates *p* < 0.05 and *p* < 0.01, Holm–Sidak *post hoc* test vs. Sleep.

SD in neocortex, neither *Arc* nor *Homer1a* (*N.S.*, Holm–Sidak *post hoc* test) levels were significantly altered by SD in any region of dorsal hippocampus (**Figure 2.5C**). *Cfos* was increased significantly with SD in CA3 only (Sleep = 2.8 ± 0.5 vs. SD = 10.7 ± 1.4 dots/mm^2, *p* < 0.01) with no significant changes in CA1 or DG (*N.S.*, Student's t-test). We then quantified transcript abundance within PV+ interneurons, using *Pvalb* mRNA expression to define the PV+ ROI (**Figure 2.4C**). Overall IEG expression in PV+ interneurons was relatively low. SD caused no significant changes in *Arc* or *Homer1a* in any layer of the neocortex, although *Cfos* dots/μm^2 increased selectively in PV+ interneurons in layer 2/3 (Sleep = 0.014 ± 0.002 vs. SD = 0.043 ± .009 dots/μm^2, *p* < 0.01). Because many PV+ interneurons expressed no detectable IEGs, we also quantified expression within the subpopulation of PV+ interneurons which had detectable levels of mRNA expression. Using a semi-automated protocol for this more circumscribed analysis, we found that SD did not affect expression levels for *Arc* or *Cfos*, but did increase *Homer1a* dots/μm^2 when measured across the entire neocortex (**Figure 2.4C**). Consistent with limited ribosome-associated transcript changes in hippocampus with SD (**Figures 2.2** and **2.3**), no significant changes in IEG expression were observed in PV+ interneurons any region of dorsal hippocampus with SD, regardless of method for quantification (**Figure 2.5D-E**).

One possibility is that the relative proportion of IEG+ PV+ interneurons varied as a function of SD. Because PV+ interneurons varied substantially in terms of ROI size, we quantified the IEG+ proportion of PV+ interneurons in Sleep and SD mice, as a function of both cell count and ROI area (**Figure 2.6**). We found the SD significantly increased the
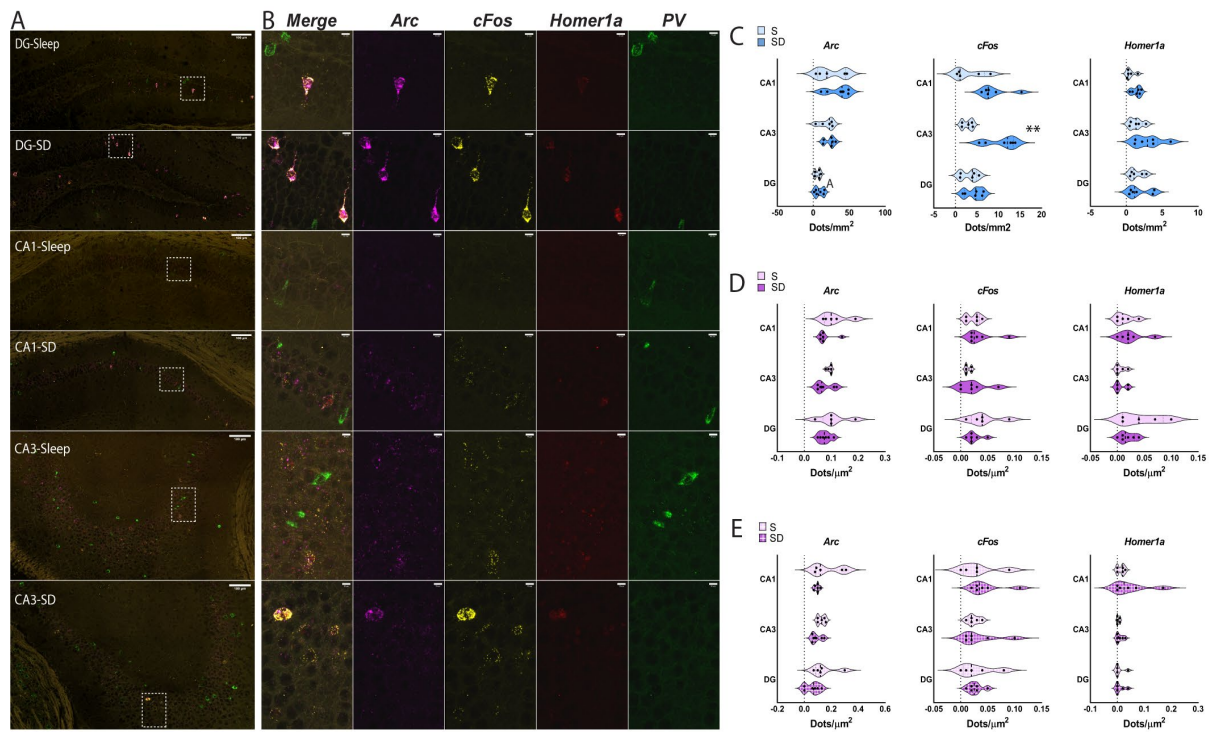
Figure 2.5. **Cell type- and region-specific changes in hippocampal IEG expression after SD.**
(A) Representative images show IEG expression in DG, CA1, and CA3 after 6 h of *ad lib* sleep ($n =$ 5 mice) or SD ($n =$ 6 mice). Inset regions are shown at higher magnification on right. Scale bars indicate 100 µm and 10 µm respectively. **(B)** 6-h SD significantly increased *Cfos* dots/mm^2 among non-PV+ (blue) cells in CA3; no other significant changes observed. **(C-D)** No significant changes were observed within DG, CA3, or CA1 in *Pvalb+* cells (magenta) **(C)** or IEG+ *Pvalb+* cells (magenta, box pattern) **(D)**. Analysis circumscribed to non-PV ROI (without *Pvalb* expression) and PV ROI (with *Pvalb* expression).Violin plots show distribution of individual subjects; ** indicates $p < 0.01$, Holm–Sidak *post hoc* test vs. Sleep.

proportion of *Arc*+ and *Cfos*+ PV+ interneurons in the neocortex, across all layers quantified (**Figure 2.6B**). No significant differences were observed in the proportion of *Homer1a*+ PV+ interneurons. Similarly, we found significant increases in the proportion of *Arc*+ and *Cfos*+ PV+ area after SD for all neocortical layers, with the exception of layer 5 (**Figure 2.6C**). No differences were observed for *Homer1a*+ area with PV+ interneurons using this measure. No significant changes in any of the mRNAs' expression were observed after SD in PV+ interneurons in any region of the hippocampus after SD, regardless of the method of quantification (**Figure 2.6D-F**).

Critically, *Pvalb* expression itself can be regulated as a function of synaptic plasticity (Donato et al., 2013). We found that when expression values were calculated cell by cell, *Pvalb* levels did vary in both DG and neocortex as a function of SD (values plotted as cumulative distributions in **Figure 2.7**). These changes moved in opposite directions, with DG neurons showing SD-driven decreases in *Pvalb* labeling intensity (**Figure 2.7A**), and neocortex showing SD-driven increases in *Pvalb* (**Figure2.7D**). However, mean *Pvalb* intensity values (calculated per area) were not affected by SD in either IEG+ PV+ interneurons or IEG- PV+ interneurons, in any structure (**Figure S3**).

Together these data suggest that SD drives relatively modest changes in *Homer1a*, *Arc*, and *Cfos* in neocortical PV+ interneurons, but does not affect these transcripts in hippocampal PV+ interneurons, and that SD drives differential hippocampal vs. neocortical changes in expression of *Pvalb*.
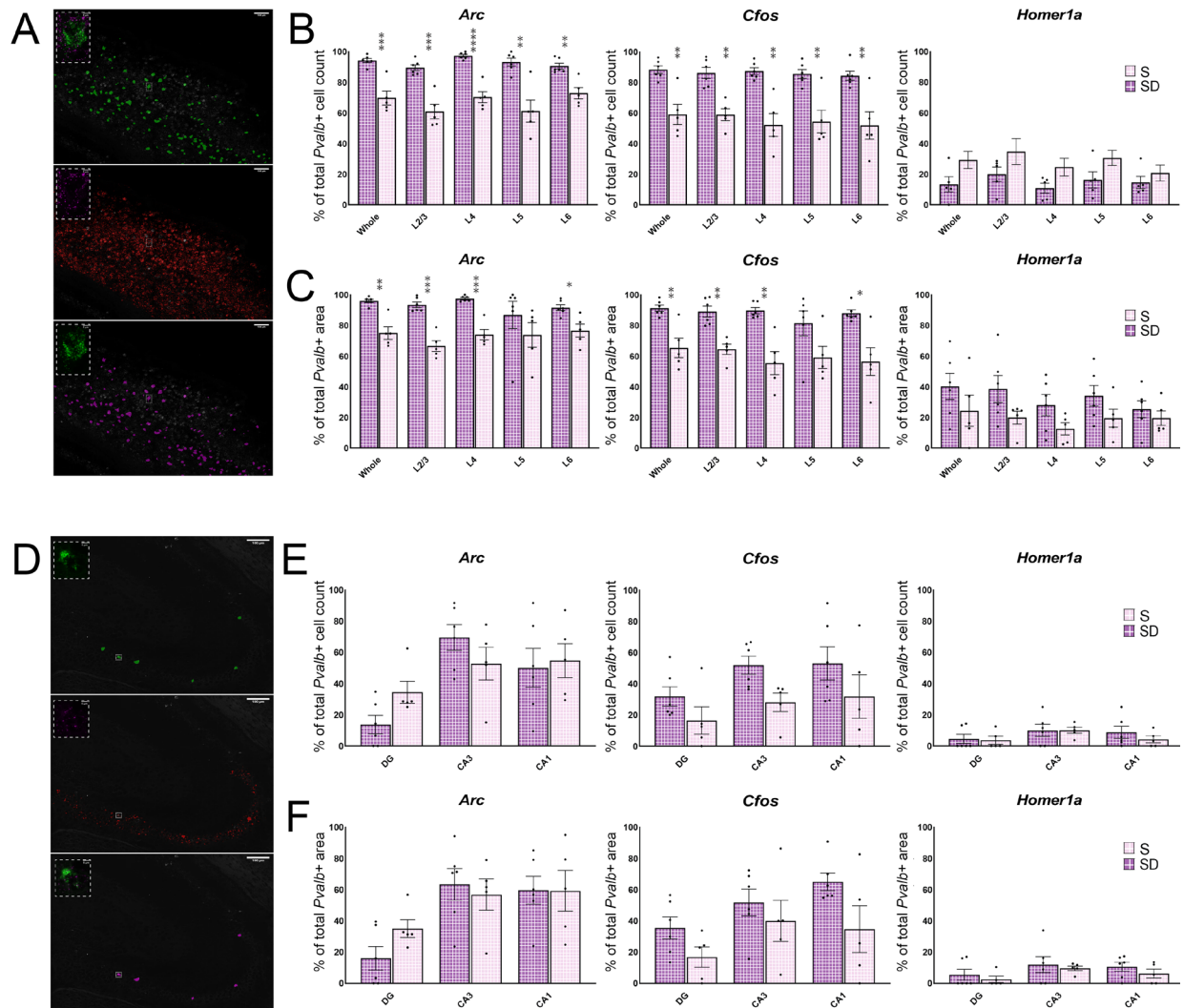
Figure 2.6. SD increases the proportion of IEG+ PV+ interneurons in neocortex, but not hippocampus. (A) An automated protocol identified *Pvalb* (green) and IEG (red) *in situ* fluorescence; cells with overlapping fluorescence were marked as IEG+ (magenta). Total IEG+ *Pvalb*+ area was then calculated as the proportion of total *Pvalb*+ area. **(B-C)** 6-h SD increased the proportion **(B)** and area **(C)** of *Pvalb*+ cells expressing *Arc* or *Cfos*, but not *Homer1a*, across most neocortical layers. Values indicate mean ± SEM; *, **, ***, and **** indicate $p < 0.05$, $p < 0.01$, $p < 0.001$, and $p < 0.0001$, respectively, Holm–Sidak post hoc test vs sleep. **(D)** The same method identified IEG+ *Pvalb*+ cells within hippocampal subregions DG, CA1, and CA3. **(E-F)** SD had no effect on the proportion **(E)** or area **(F)** of *Pvalb*+ hippocampal cells expressing *Arc*, *Cfos*, or *Homer1a*, Values indicate mean ± SEM; *N.S.*, Holm–Sidak *post hoc* test vs. Sleep.
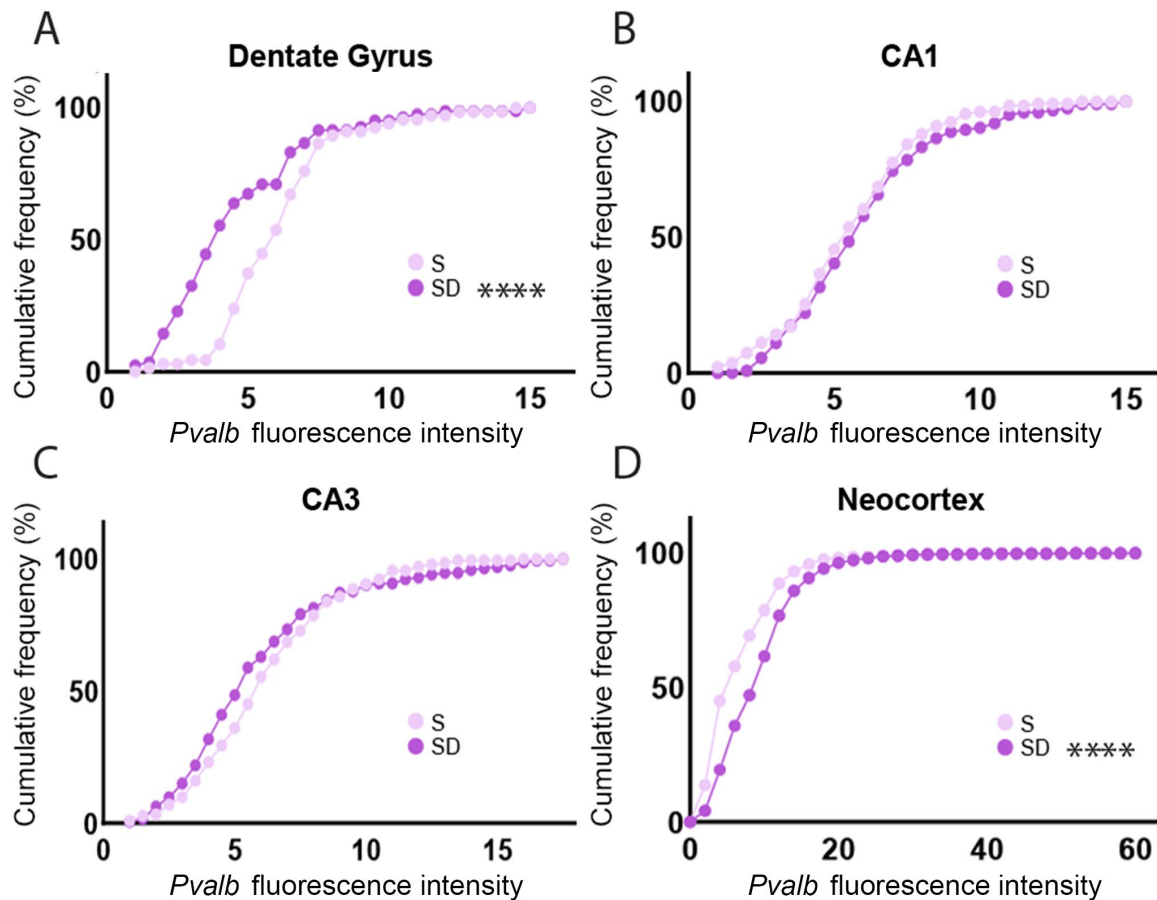
Figure 2.7. **SD alters neuronal *Pvalb* mean fluorescence intensity in a region- and subregion-specific manner.** Cumulative frequency distributions showing the impact of 6-h SD on *Pvalb* mean fluorescence intensity in individual *Pvalb*+ cells of the hippocampus and neocortex**. (A)** 6-h SD significantly decreased mean fluorescence intensity of *Pvalb* within *Pvalb*+ cells of the DG while having no significant effect on **(B)** CA1 or **(C)** CA3 intensity. **(D)** 6-h SD significantly increased mean fluorescence intensity of *Pvalb* within *Pvalb*+ cells of the neocortex. Hippocampal (DG, CA1, CA3) and neocortical bin widths for cumulative frequency distributions set at 0.5 and 2 respectively; **** indicates $p < 0.0001$, Holm–Sidak *post hoc* test vs. Sleep.

2.8 Results: Cell type- and region-specific effects of SD on ribosome-associated transcripts involved in circadian timekeeping

SD has previously been implicated in regulating core molecular clock genes' expression. As is true for IEG expression, the extent to which SD differentially impacts core clock gene expression as a function of cell type and regions is unclear. Consequently, we quantified ribosome-associated transcript abundance for core clock genes- *Clock, Per1, Per2, Cry1, Cry1, and Bmal1-* after SD in Camk2a+ neurons and PV+ interneurons of the neocortex and hippocampus (**Figure 2.8**). Consistent with findings from whole neocortical tissue (Franken et al., 2007; Hoekstra et al., 2019), we found that 3-h SD significantly increased *Per2* expression in neocortical Camk2a+ neurons and PV+ interneurons (**Figure 2.8A**). In contrast, SD had no significant impact on transcript abundance in the hippocampus of either population. Longer-duration (6-h) SD resulted in no further changes in neocortical transcript abundance (with *Per2* levels tending to remain elevated in both Camk2a+ neurons and PV+ interneurons) (**Figure 2.8B**). Within the hippocampus, 6-h SD significantly altered abundance of ribosome-associated *Per2, Cry1*, and *Cry2* transcripts in Camk2a+ neurons (increasing *Per2* and *Cry1*, decreasing *Cry2*), while having no significant effect on transcript abundance in PV+ We also quantified (after SD vs Sleep) the abundance of ribosome-associated mRNAs encoding other cellular timekeeping components: *Rev-Erbα, Dbp, Ted, Nfil3, and Dec1* (**Figure 2.9**). We found significant heterogeneity in how these auxiliary clock genes responded to SD in different cell types and regions. None of the transcripts were significantly altered in either cell type in the hippocampus, with either 3-h or 6-h SD
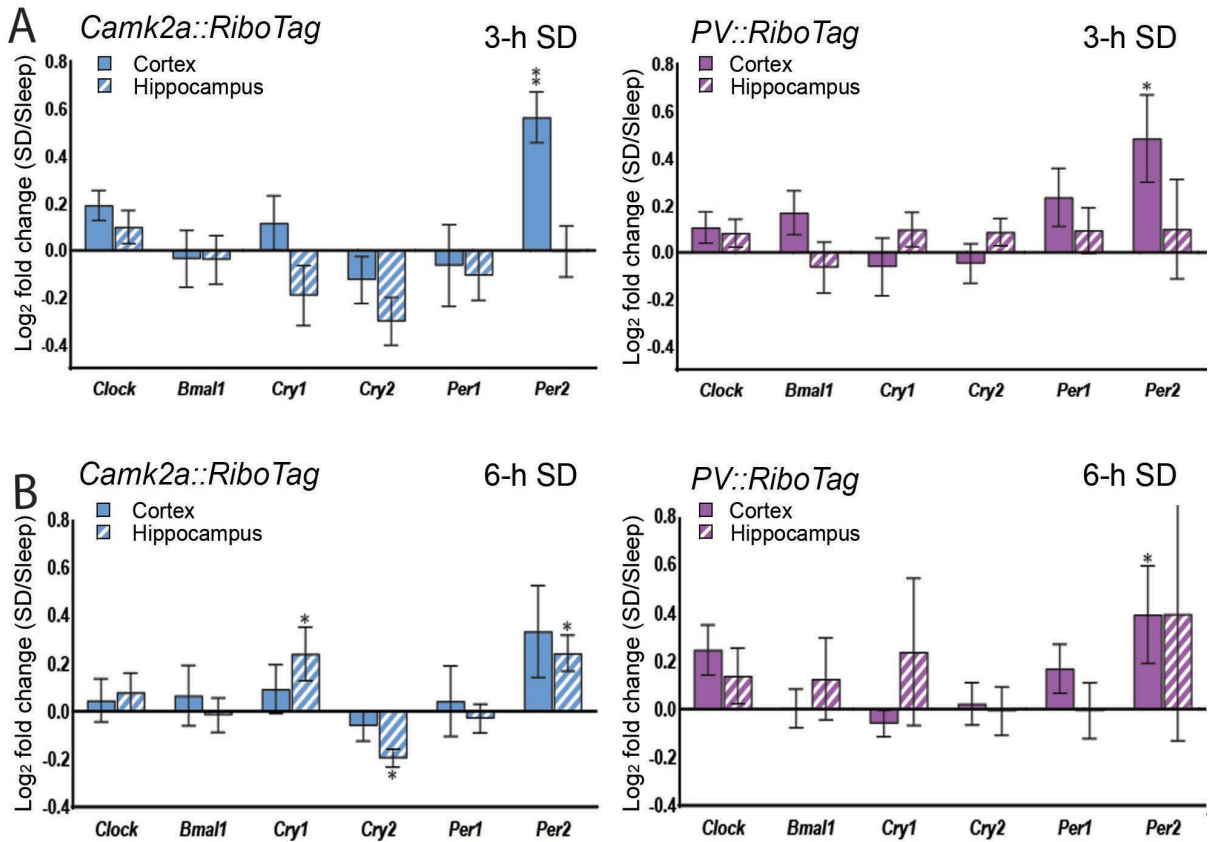
Figure 2.8. **SD alters ribosome-associated transcripts encoding core clock genes in a cell type and region-specific manner**. **(A)** 3-h SD significantly increased *Per2* abundance on ribosomes in Camk2a+ (blue) and PV+ (magenta) neocortical neurons; no significant changes in core clock transcripts were observed in hippocampal neurons. **(B)** After 6-h SD, *Per2* abundance remained significantly elevated in neocortical PV+ interneurons. Ribosome-associated *Cry1*, *Cry2*, and *Per2* were all altered after 6-h SD in the hippocampal Camk2a+ neuron population. No significant change observed among PV+ interneurons. Transcript level changes are presented as a $\log_2$ fold change between SD and *ad lib* sleep mice. All statistical tests were done on ΔCT values. Values indicate mean ± SEM with propagated error from *ad lib* sleep and SD groups ; * and ** indicate $p < 0.05$ and $p < 0.01$, respectively, Holm–Sidak *post hoc* test vs. Sleep.
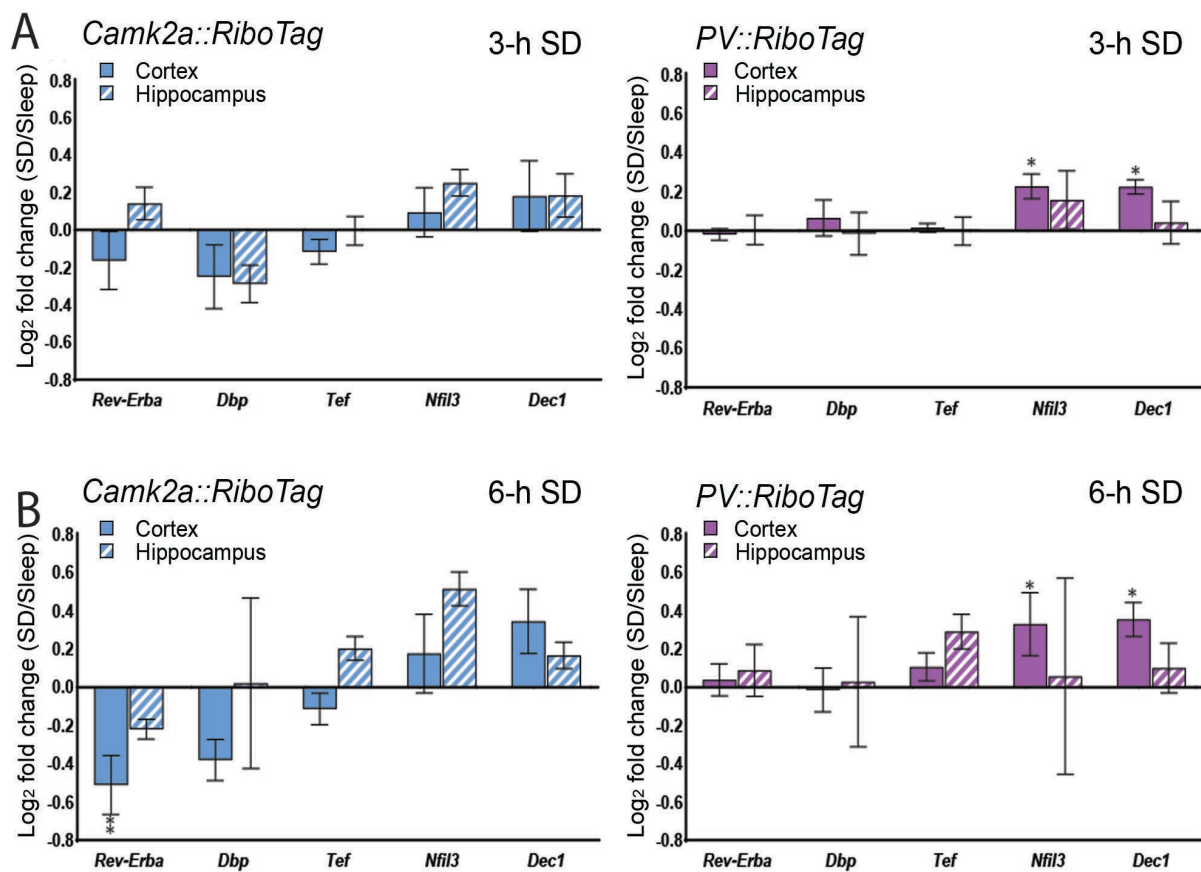
Figure 2.9. SD differentially alters circadian clock modifiers in Camk2a+ and PV+ neocortical populations. (A) 3-h SD had no significant effect on ribosome-associated circadian clock modifier transcripts among Camk2a+ (blue) neurons in neocortex, but increased *Nfil3* and *Dec1* expression among neocortical PV+ interneurons (magenta). **(B)** 6-h SD significantly decreased *Rev-Erbα* abundance on ribosomes in Camk2a+ neocortical neurons. No transcripts were significantly altered by SD in either neuron population in hippocampus. Transcript level changes are presented as a $\log_2$ fold change between SD and *ad lib* sleep mice. All statistical tests were done on ΔCT values. Values indicate mean ± SEM with propagated error from *ad lib* sleep and SD groups; * and ** indicate $p < 0.05$ and $p < 0.01$, respectively, Holm–Sidak *post hoc* test vs. Sleep.

(**Figure 2.9A-B**). However, within the neocortex, both 3-h and 6-h SD significantly increased cortical *Nfil3* and *Dec1* abundance in PV+ interneurons. While these transcripts were not significantly altered in neocortical Camk2a+ neurons, 6-h SD significantly decreased *Rev-Erbα* expression in Camk2a+ neocortical neurons (**Figure 2.9B**).

2.9 Discussion:

Here, using TRAP, we have identified SD-driven molecular changes unique to specific cell populations in hippocampus and neocortex. Numerous studies have used transcriptome (Cirelli et al., 2004; Vecsey et al., 2012) or proteome (Noya et al., 2019; Poirrier et al., 2008) profiling of these structures following sleep vs. SD as a way of clarifying the functions of sleep in the brain. We find that comparing across structures, there are large differences in SD effects on ribosome-associated transcripts. For example, while even brief (3-h) SD increases abundance of plasticity-mediating transcripts in neocortical Camk2a+ neurons and PV+ interneurons (**Figure 2**) few of these transcripts are altered in hippocampus even after longer SD. This is particularly true for hippocampal PV+ interneurons, for which none of the transcripts are significantly altered by SD. SD-driven changes in abundance for IEG transcription regulators follow a similar pattern (**Figure 2.3**), with hippocampal PV+ interneurons in particular being refractory to SD. Our *in situ* analysis of mRNA abundance in PV+ and PV- neurons (**Figures 2.4-2.6**) is consistent with this interpretation, and suggests that even within neocortex, SD-driven changes in these transcripts' abundance are relatively modest in PV+ interneurons (**Figure 2.4**).

While IEGs are generally assumed to reflect specific patterns of recent neuronal activity (Tyssowski and Gray, 2019), there are brain region- and microcircuit-specific differences in IEG expression which reflect neurons' network connectivity patterns (Gonzalez et al., 2019; Tyssowski et al., 2018). Moreover, IEG expression in PV+ interneurons is regulated by distinct cellular pathways and is differentially gated by neuronal activation (Cohen et al., 2016). Indeed, some studies have failed to detect IEGs in PV+ interneurons altogether (Imamura et al., 2011; Vazdarjanova et al., 2006), and our present results showing relatively low expression in the PV+ interneuron population (**Figures 2.4-2.6**). However, insofar as abundance of all of these transcripts is regulated by neuronal activity to some degree (Donato et al., 2013; Yap and Greenberg, 2018), our present data support two broad conclusions. First, neuronal activation in the hippocampus is reduced relative to neocortex during SD. Second, PV+ interneuron activity may vary less as a function of SD than Camk2a+ neuron activity.

The former conclusion has major implications for the field of learning and memory, where pronounced and selective effects of sleep disruption on hippocampal processes (e.g., episodic and spatial memory consolidation) have been well described (Havekes and Abel, 2017; Puentes-Mestril et al., 2019; Saletin and Walker, 2012). In hippocampal structures such as the DG and CA1, available data suggest that both markers of neuronal activity and synaptic plasticity are disrupted after SD (Delorme et al., 2019; Havekes et al., 2016; Ognjanovski et al., 2018; Raven et al., 2019; Tudor et al., 2016). Our present data largely confirm these findings, and suggest that particularly in DG and CA1 (**Figure 2.5**), there is little evidence of neuronal activation during SD. Indeed, we find that DG

neurons show decreased *Pvalb* expression after SD, while neocortical neurons simultaneously show increased expression (**Figure 2.7**). Critically, *Pvalb* expression levels have been shown to correlate with both PV+ interneuron activity level and the relative amounts of excitatory to inhibitory input PV+ interneurons receive (Donato et al., 2013). Thus we conclude that SD increases excitatory input to PV+ interneurons in neocortex, while simultaneously decreasing excitatory input to DG. This conclusion parallels our recent work showing differential effects of SD on another activity marker, *Arc*, in DG vs. neocortex, and suggests that SD may have a uniquely disruptive effect on network activity in DG.

The latter conclusion also has important implications for maintenance of excitatory-inhibitory (E-I) balance during SD. Recent data suggest that E-I balance normally varies over the course of the day, in a sleep-dependent manner (Bridi et al., 2020). Furthermore, prior evidence from both whole-tissue transcriptome profiling and immunohistochemistry has suggested that SD may differentially affect connections from excitatory to inhibitory neurons (and vice versa) in structures like the neocortex (Del Cid-Pellitero et al., 2017; Puentes-Mestril and Aton, 2017). Because sleep loss is one of the major risk factors for triggering seizure onset in epilepsy (Frucht et al., 2000; Lawn et al., 2014), an underlying mechanism might be differential activation of, or plasticity in, interneurons vs. principal neurons with SD. Interactions between PV+ interneurons and principal neurons are particularly important in both regulation of attention (Aton, 2013) and in generating network oscillations important for memory consolidation (Ognjanovski et al., 2018; Ognjanovski et al., 2017). Insofar as SD may disrupt both attention and memory

consolidation, differential effects on activity of PV+ and Camk2a+ neurons in the hippocampus and neocortex may be an important underlying mechanism.

Because many of the transcripts quantified here (e.g., *Arc*, *Homer1a*, *Narp*, and *Bdnf*) play a critical role in activity-regulated synaptic plasticity, the fact that their abundance on translating ribosomes in Camk2+ and PV+ neurons is differentially altered by SD (**Figure 2.2**) also has intriguing implications. For example, it suggests that SD could lead to long-lasting changes in the E-I balance and information processing capacity of neocortical and hippocampal circuits. This may be a plausible mechanism for some of the reported longer-lasting brain metabolic (Wu et al., 2006) and cognitive (Belenky et al., 2003; Chai et al., 2020; Dinges et al., 1997) effects of SD (i.e., those that do not normalize with recovery sleep).

Alterations in brain clock gene expression with SD has been widely reported (Franken et al., 2007; Mongrain et al., 2011; Wisor et al., 2002; Wisor et al., 2008). Along with transcripts such as *Homer1a* (Maret et al., 2008; Zhu et al., 2020), SD-driven increases in transcripts such as *Per2* are hypothesized to play a role in homeostatic aspects of sleep regulation (Franken et al., 2007; Mang and Franken, 2015). Our data suggest that similar to plasticity-regulating transcripts (including *Homer1a*), SD-mediated changes in clock gene transcripts on ribosomes are cell type- and brain region-specific (**Figures 8** and **9**). For example, while *Per2* increases on both Camk2a+ and PV+ neocortical neuron-derived ribosomes with as little as 3 h SD, no clock gene transcripts are altered in the hippocampus with 3-h SD (**Figure 2.8A**, **Figure 2.9A**). Another example

is *Rev-erbα*, which is significantly reduced after 6-h SD, but only in neocortical Camk2a+ neurons. An interesting and important issue, raised by our findings, is that SD-driven changes in particular core clock transcripts' abundance do not move in the same direction, as they normally would during a 24-h cycle (e.g., *Cry1*, *Cry2*, and *Per2*; **Figure 2.8**). This suggests that SD-driven changes in these transcripts may not be driven by canonical E-box elements, consistent with recent findings (Mongrain et al., 2011). However, because changes in these transcripts may have numerous downstream effects on transcription of other clock-control genes (Chiou et al., 2016; Schmutz et al., 2010), these SD-driven changes may have even more numerous downstream effects that changes in plasticity effectors' transcripts. Future studies will be needed to quantify longer-term cell type-specific changes to physiology and structure initiated during SD, and the molecular events responsible for these changes.

Together our data suggest that effects of SD on plasticity, timekeeping, and homeostatic regulation of brain circuitry is heterogeneous, and likely involves subtle modifications to microcircuits (e.g., those in hippocampal subregions and neocortical layers) critical for appropriate brain function.

Tables:

Table 2.1. Housekeeping pairs used for RiboTag conditions. Change in gene expression presented as ratio[1] and fold change[2].

mRNA Population	Condition	Region	Gene Pair	SD(2^{-CT})/S(2^{-CT})[1]	Fold Change[2]
Camk2a	3hrs	Cortex	Actg1/Hprt1	0.98	-1.02
		Hippocampus	Gapdh/Tuba4a	0.90	-1.11
	6hrs	Cortex	Pgk1/Tbp	0.87	-1.15
		Hippocampus	Gapdh/Tuba4a	0.92	-1.08
Parvalbumin	3hrs	Cortex	Actg1/Hprt1	0.82	-1.22
		Hippocampus	Gapdh/Tuba4a	1.02	1.02
	6hrs	Cortex	Pgk1/Tbp	0.97	-1.03
		Hippocampus	Gapdh/Tuba4a	1.02	1.02

Table 2.2. SD induced changes in ribosome-associated transcript abundance within Camk2a:RiboTag mice.

Gene Name	Region	Sleep, ΔCT		SD, ΔCT		qPCR p-value (Sleep vs. SD)		Sleep,n		SD, n	
		3-h	6-h	3-h	6-h	3-h	6-h	3-h	6-h	3-h	6-h
Arc	CTX	5.16 ± 0.37	2.20 ± 0.26	3.9 ± 0.19	0.93 ±0.17	0.0003	<0.0001	4	6	5	6
	HP	4.29 ± 0.07	5.05 ± 0.09	3.98± .15	4.53 ± 0.12	0.4663	0.0036	4	6	5	6
Homer1a	CTX	5.11 ± 0.29	2.25 ± 0.16	4.053 ± 0.18	0.44 ± 0.04	0.0017	<0.0001	4	6	5	6
	HP	5.97 ± 0.09	5.33 ± 0.74	5.12 ±.232	3.76 ± 0.04	0.0036	<0.0001	4	6	5	6
Narp	CTX	5.34 ± 0.05	2.00 ± 0.10	4.861 ± 0.08	1.66±.12	0.1688	0.0902	4	6	5	6
	HP	6.09 ± 0.13	4.932 ± 0.16	5.98 ±.21	4.86 ± 0.15	0.8637	0.7475	4	6	5	6
Bdnf	CTX	5.02 ± 0.12	1.54 ± 0.06	4.542 ± .10	.90 ± 0.07	0.1688	0.0049	4	6	5	6
	HP	3.94 ± 0.03	4.09 ± 0.10	3.92 ± .16	3.99 ± 0.07	0.9288	0.7475	4	6	5	6
Npas4	CTX	8.42 ± 0.23	4.34 ± 0.12	7.64 ± 0.20	3.50 ± 0.10	0.1370	0.0031	4	6	5	6
	HP	8.61 ± 0.38	8.58 ± 0.18	8.87 ± 0.14	7.80 ± 0.12	0.4478	0.0005	4	6	5	6
cFos	CTX	6.17 ± 0.42	3.11 ± 0.31	5.68 ± 0.24	1.81 ± 0.17	0.2664	<0.0001	4	6	5	6
	HP	7.64 ± 0.15	8.32 ± 0.13	6.74 ± 0.20	6.52 ± 0.18	0.0209	<0.0001	4	6	5	6
FosB	CTX	5.99 ± 0.12	2.87 ± 0.21	5.42 ± 0.26	1.98 ± 0.10	0.2664	0.0031	4	6	5	6
	HP	6.45 ± 0.09	6.69 ± 0.13	6.10 ± 0.22	5.71 ± 0.07	0.4478	<0.0001	4	6	5	6
Clock	CTX	3.95 ± 0.07	0.97 ± 0.08	3.76 ± 0.02	0.93 ± 0.04	0.6176	0.9770	4	6	5	6
	HP	3.45 ± 0.07	3.63 ± 0.06	3.35 ± 0.05	3.56 ± 0.06	0.8666	0.6647	4	6	5	6
Bmal1	CTX	4.68 ± 0.11	1.34 ± 0.10	4.71 ± 0.09	1.27 ± 0.07	0.8745	0.9770	4	6	5	6
	HP	4.35 ± 0.09	4.37 ± 0.05	4.39 ± 0.08	4.38 ± 0.06	0.9378	0.9028	4	6	5	6
Cry1	CTX	5.90 ± 0.09	2.33 ± 0.08	5.78 ± 0.10	2.24 ± 0.07	0.8434	0.9592	4	6	5	6
	HP	5.88 ± 0.10	6.34 ± 0.10	6.07 ± 0.11	6.10 ± 0.05	0.5022	0.0138	4	6	5	6
Cry2	CTX	4.23 ± 0.03	0.36 ± 0.05	4.36 ± 0.11	0.42 ± 0.04	0.8434	0.977	4	6	5	6
	HP	3.80 ± 0.02	3.75 ± 0.04	4.10 ± 0.11	3.95 ± 0.01	0.1126	0.0493	4	6	5	6
Per1	CTX	3.90 ± 0.16	0.23 ± 0.10	3.97 ± 0.13	0.19 ± 0.11	0.8745	0.9770	4	6	5	6
	HP	3.62 ± 0.05	3.31 ± 0.03	3.73 ± 0.11	3.34 ± 0.05	0.8666	0.9028	4	6	5	6
Per2	CTX	7.43 ± 0.08	3.71 ± 0.18	6.87 ± 0.10	3.38 ± 0.06	0.0012	0.0654	4	6	5	6
	HP	6.62 ± 0.07	6.80 ± 0.07	6.62 ± 0.10	6.56 ± 0.04	0.9744	0.0138	4	6	5	6
Rev-Erba	CTX	3.32 ± 0.16	-0.50 ± .14	3.48 ± 0.09	0.02 ± 0.06	0.7553	0.0066	4	6	5	6
	HP	4.05 ± 0.09	1.92 ± 0.05	3.91 ± 0.05	2.13 ± 0.03	0.3593	0.7601	4	6	5	6
Dbp	CTX	5.29 ± 0.15	2.47 ± 0.08	5.54 ± 0.13	2.85 ± 0.07	0.5635	0.0570	4	6	5	6
	HP	5.33 ± 0.07	4.05 ± 0.24	5.61 ± 0.09	4.03 ± 0.37	0.0583	0.9171	4	6	5	6
Tef	CTX	3.47 ± 0.02	0.62 ± 0.07	3.58 ± 0.07	0.74 ± 0.04	0.7553	0.4556	4	6	5	6
	HP	3.14 ± 0.02	2.54 ± 0.05	3.14 ± 0.08	2.33 ± 0.04	0.9679	0.7601	4	6	5	6
Nfil3	CTX	6.34 ± 0.14	3.80 ± 0.19	6.29 ± 0.07	3.62 ± 0.07	0.7553	0.4310	4	6	5	6
	HP	6.19 ± 0.07	5.45 ± 0.05	5.94 ± 0.04	4.94 ± 0.07	0.0997	0.083	4	6	5	6
Dec1	CTX	3.36 ± 0.16	-0.43 ± 0.15	3.18 ± 0.15	-0.77 ± 0.08	0.7553	0.0754	4	6	5	6
	HP	2.74 ± 0.06	1.37 ± 0.05	2.55 ± 0.11	1.21 ± 0.05	0.2666	0.7601	4	6	5	6

Table 2.3. SD induced changes in ribosome-associated transcript abundance within PV:RiboTag mice.

Gene Name	Region	Sleep, ΔCT		SD, ΔCT		qPCR p-value (Sleep vs. SD)		Sleep,n		SD, n	
		3-h	6-h	3-h	6-h	3-h	6-h	3-h	6-h	3-h	6-h
Arc	CTX	6.64 ± 0.23	6.87 ± 0.07	5.25 ± 0.43	5.40 ± 0.07	0.0002	<0.0001	4	5	4	6
	HP	6.67 ± 0.09	5.46 ± 0.14	6.58 ± 0.11	5.29 ± .19	0.9758	0.8837	4	6	5	6
Homer1a	CTX	7.49 ± 0.08	6.71 ± 0.13	7.03 ± 0.10	5.27 ± 0.12	0.3025	<0.0001	4	5	4	6
	HP	9.02 ± 0.23	7.32 ± 0.35	9.00 ± 0.23	7.04 ± 0.27	0.9758	0.8673	4	6	5	6
Narp	CTX	8.22 ± 0.08	7.39 ± 0.07	7.77 ± 0.13	7.01 ± 0.09	0.3025	0.0196	4	5	4	6
	HP	8.60 ± 0.15	8.58 ± 0.47	8.71 ± 0.19	8.22 ± 0.21	0.9758	0.8383	4	6	5	6
Bdnf	CTX	8.03 ± 0.12	7.36 ± 0.19	7.89 ± 0.14	6.79 ± 0.09	0.6257	0.0014	4	5	4	6
	HP	0.60 ± 0.21	5.38 ± 0.26	1.17 ± 0.27	5.44 ± 0.20	0.2158	0.8907	4	6	5	6
Npas4	CTX	9.14 ± 0.43	7.97 ± 0.13	7.96 ± 0.14	7.11 ± 0.10	0.0143	0.0008	4	5	4	6
	HP	9.15 ± 0.20	5.21 ± 0.31	9.36 ± 0.13	5.40 ± 0.12	0.2737	0.6749	4	6	5	6
cFos	CTX	7.81 ± 0.10	7.04 ± 0.13	6.85 ± 0.38	5.21 ± 0.13	0.0336	<0.0001	4	5	4	6
	HP	9.81 ± 0.13	7.56 ± 0.32	9.51 ± 0.10	5.98 ± 0.18	0.2081	0.0042	4	6	5	6
FosB	CTX	7.93 ± 0.16	7.05 ± 0.17	7.68 ± 0.15	6.22 ± 0.20	0.5072	0.0008	4	5	4	6
	HP	10.86 ± 0.12	6.65 ± 0.45	10.42 ± 0.11	7.10 ± 0.40	0.0814	0.5437	4	6	5	6
Clock	CTX	3.58 ± 0.07	3.29 ± 0.09	3.47 ± 0.04	3.04 ± 0.06	0.8513	0.2076	4	5	4	6
	HP	4.11 ± 0.05	4.06 ± 0.58	4.02 ± 0.05	2.68 ± 0.08	0.9716	0.9770	4	6	5	6
Bmal1	CTX	5.42 ± 0.09	4.51 ± 0.08	5.25 ± 0.07	4.51 ± 0.04	0.6893	0.9848	4	5	4	6
	HP	5.86 ± 0.09	3.71 ± 0.56	5.93 ± 0.09	5.59 ± 0.12	0.9716	0.9770	4	6	5	6
Cry1	CTX	8.57 ± 0.08	4.45 ± 0.03	8.63 ± 0.13	4.51 ± 0.05	0.8942	0.9436	4	5	4	6
	HP	6.24 ± 0.04	3.98 ± 0.60	6.15 ± 0.07	4.22 ± 0.23	0.9716	0.9099	4	6	5	6
Cry2	CTX	6.37 ± 0.05	3.12 ± 0.07	6.42 ± 0.09	3.09 ± 0.06	0.8942	0.979	4	5	4	6
	HP	5.18 ± 0.05	3.63 ± 0.61	5.09 ± 0.04	2.82 ± 0.07	0.9716	0.9994	4	6	5	6
Per1	CTX	4.16 ± 0.12	2.61 ± 0.10	3.92 ± 0.09	2.45 ± 0.04	0.4605	0.5214	4	5	4	6
	HP	4.66 ± 0.11	3.57 ± 0.53	4.56 ± 0.05	2.17 ± 0.08	0.9716	0.9994	4	6	5	6
Per2	CTX	7.70 ± 0.23	6.34 ± 0.15	7.21 ± 0.03	5.95 ± 0.15	0.0121	0.0113	4	5	4	6
	HP	6.98 ± 0.23	4.46 ± 0.89	6.88 ± 0.11	5.05 ± 0.24	0.9716	0.6197	4	6	5	6
Rev-Erba	CTX	3.89 ± 0.03	2.51 ± 0.06	3.91 ± 0.02	2.47 ± 0.07	0.9536	0.9276	4	5	4	6
	HP	4.97 ± 0.07	2.34 ± 0.09	4.96 ± 0.06	2.26 ± 0.11	0.9993	0.9805	4	6	5	6
Dbp	CTX	6.42 ± 0.07	5.47 ± 0.10	6.35 ± 0.09	5.49 ± 0.07	0.7058	0.9276	4	5	4	6
	HP	7.62 ± 0.08	6.12 ± 0.23	7.63 ± 0.10	6.10 ± 0.25	0.9993	0.9805	4	6	5	6
Tef	CTX	3.98 ± 0.02	3.35 ± 0.06	3.96 ± 0.02	3.24 ± 0.05	0.9536	0.7245	4	5	4	6
	HP	4.80 ± 0.09	3.23 ± 0.05	4.80 ± 0.02	2.94 ± 0.07	0.9993	0.7872	4	6	5	6
Nfil3	CTX	7.11 ± 0.05	6.46 ± 0.17	6.88 ± 0.06	6.13 ± 0.07	0.0102	0.0206	4	5	4	6
	HP	7.86 ± 0.10	8.58 ± 0.47	7.71 ± 0.13	8.22 ± 0.21	0.6950	0.713	4	6	5	6
Dec1	CTX	4.88 ± 0.04	3.62 ± 0.09	4.66 ± 0.02	3.27 ± 0.04	0.0102	0.0143	4	5	4	6
	HP	5.54 ± 0.11	3.92 ± 0.11	5.49 ± 0.07	3.82 ± 0.07	0.9951	0.9805	4	6	5	6

2.10 Supplementary material:

Table 2.4. Primer Designs

Gene Name	Forward Primer	Reverse Primer
Actg1	ACTCTTCCAGCCTTCCTTC	ATCTCCTTCTGCATCCTGTC
Hprt	CGTGATTAGCGATGATGAACCA	CTTTCAGTCCTGTCCATAATCAGT
Gapdh	GTGTTTCCTCGTCCCGTAGA	AATCCGTTCACACCGACCTT
Pgk1	TCGTGATGAGGGTGGACTTC	ACAGCAGCCTTGATCCTTTG
Cypa	CCACCGTGTTCTTCGACATC	AGGAACCCTTATAGCCAAATCCT
Tuba4a	ATGCGCGAGTGCATTTCAG	CACCAATGGTCTTATCGCTGG
Tbp	GCAGCCTCAGTACAGCAATC	GGTGCAGTGGTCAGAGTTTG
Arc	CCAGATCCAGAACCACATGAA	GAGAGTGTACCCTCACTGTATTG
Homer1a	GCATTGCCATTTCCACATAGG	ATGAACTTCCATATTTATCCACCTTACTT
Narp	TGCTGATAGAGTGGGGCAAT	CAGCTGTGCGACCTTGTC
Bdnf	GGTCACAGCGGCAGATAAA	TCAGTTGGCCTTTGGATACC
Npas4	CTTCTCAACACTACCGCCTG	TGCTTGGCTTGAAGTCTCAC
cFos	GAAGAGGAAGAGAAACGGAGAAT	CTTGGAGTGTATCTGTCAGCTC
FosB	AGAAGACCCCGAGAAGAGAC	TCTTCGTAGGGGATCTTGCA
Clock	CCAAAGGCCAGCAGTGGATA	TTGTCAGCAGCTGTCTCAGG
Bmal1	CCCATACACAGAAGCAAACT	ACAGACTCGGAGACAAAGAG
Cry1	TCCCCTCCCCTTTCTCTTTA	TTGTCCCAAGGGATCTGAAC
Cry2	AAGCTGAATTCGCGTCTGTT	AACAGCCTTGGGAACACATC
Per1	CCAGGATGTGGGTGTCTTCT	TTTCCTGGGTGAAGTCCTTG
Per2	AAGAACGCGGATATGTTTGC	CAGGATCTTCCCAGAAACCA
Rev-Erba	CGACCCTGGACTCCAATAACA	AACCTTGAGTCAGGGACTGG
Dbp	GCCAGCTGCTTGACATCTAGG	GCATCTCTCGACCTCTTGGC
Tef	CCTTCCCTCTGGTCCTGAAGA	CAGAGACGGCCATGGTACTG
Nfil3	GAGGGTGTAGTGGGCAAGTC	ATCCGAAGCTTGTGCGGTAA
Dec1	TGGCGAAGCATGAGAACACT	CTTTGGGAGCCGAGTCCAAT
Mbp	CCTTGACTCCATCGGGCGCT	CTTCTGGGGCAGGGAGCCAT
Gfap	TCCTGGAACAGCAAAACAAG	CAGCCTCAGGTTGGTTTCAT
NPY	CAAGAGATCCAGCCCTGAGA	ACATGGAAGGGTCTTCAAGC
SOM	CTCGGACCCCAGACTCCGTC	CTCGGGCTCCAGGGCATCAT
Griar4	GTTTTCCCTGGGTGCCTTTA	GAAGAACCACCATACGCCTC
Gad67	GACACCGGGGACAAGGCGAT	TCCCACGGTGCCCTTTGCTT
Parvalbumin	GTCGATGACAGACGTGCTCA	TTGTGGTCGAAGGAGTCTGC
Vglut2	ATACTAGAGGGGTGGCCATC	GTGCAGCAATGAGGAAGACA
Vglut1	CCAGCATCTCTGAGGAGGAG	GGCTGAGAGATGAGGAGCAG
Camk2a	GCCTGTACCAGCAGATCATCAAA	GGGTTGATGGTCAGCATCTTA

Table 2.5 Housekeeping stability analysis for Camk2a::RiboTag samples.

| Gene name	Condition	Region	Comprehensive		Genorm*		NormFinder		Intergroup Variation		Intragroup Variation	
			Stability	Ranking	Stability	Ranking	Stability	Ranking	S	SD	S	SD
Actg1	3-h	CTX	4.95	6	0.066	3	0.025	6	-0.005	0.005	0.003	0.003
		HIP	5.18	6	0.112	5	0.060	4	0.026	-0.026	0.003	0.005
	6-h	CTX	7	7	0.158	6	0.102	7	0.066	-0.066	0.038	0.005
		HIP	7	7	0.147	6	0.053	7	0.010	-0.010	0.020	0.014
Hprt	3-h	CTX	2.45	2	0.057	2	0.012	2	0.009	-0.009	0.000	0.002
		HIP	7	7	0.183	6	0.151	6	-0.119	0.119	0.085	0.011
	6-h	CTX	2.66	3	0.081	1	0.061	4	-0.028	0.028	0.009	0.002
		HIP	1	1	0.055	1	0.012	1	0.013	-0.013	0.000	0.002
Gapdh	3-h	CTX	1.41	1	0.048	1	0.009	1	0.010	-0.010	0.000	0.000
		HIP	1	1	0.049	1	0.037	1	0.012	-0.012	0.002	0.002
	6-h	CTX	6	6	0.127	5	0.078	6	0.060	-0.060	0.003	0.001
		HIP	4.56	5	0.126	5	0.037	6	0.013	-0.013	0.008	0.009
Pgk1	3-h	CTX	2.82	3	0.048	1	0.020	4	0.006	-0.006	0.002	0.001
		HIP	3.46	4	0.060	2	0.069	5	0.025	-0.025	0.011	0.004
	6-h	CTX	1.73	1	0.081	1	0.052	2	-0.016	0.016	0.009	0.003
		HIP	5.23	6	0.111	4	0.031	5	0.003	-0.003	0.004	0.007
Cypa	3-h	CTX	3.56	4	0.072	4	0.019	3	0.015	-0.015	0.001	0.002
		HIP	3.22	3	0.084	3	0.052	3	0.030	-0.030	0.000	0.004
	6-h	CTX	3.46	5	0.088	2	0.067	5	-0.038	0.038	0.006	0.003
		HIP	2	2	0.055	1	0.021	3	0.009	-0.009	0.005	0.001
Tuba4a	3-h	CTX	5.73	7	0.081	5	0.022	5	-0.025	0.025	0.002	0.002
		HIP	2.11	2	0.049	1	0.045	2	0.013	-0.013	0.004	0.002
	6-h	CTX	2.24	2	0.105	4	0.022	1	0.000	0.000	0.000	0.003
		HIP	4.23	4	0.089	3	0.024	4	-0.018	0.018	0.001	0.007
Tbp	3-h	CTX	4.3	5	0.085	6	0.027	7	-0.011	0.011	0.003	0.003
		HIP	4.95	5	0.099	4	0.045	2	0.013	-0.013	0.001	0.006
	6-h	CTX	2.83	4	0.096	3	0.060	3	-0.044	0.044	0.001	0.001
		HIP	3	3	0.078	2	0.020	2	-0.030	0.030	0.003	0.002

Table 2.6 Housekeeping stability analysis for PV::RiboTag samples.

Gene name	Condition	Region	Comprehensive		Genorm*		NormFinder		Intergroup Variation		Intragroup Variation	
			Stability	Ranking	Stability	Ranking	Stability	Ranking	S	SD	S	SD
Actg1	3-h	CTX	5.12	5	0.089	6	0.033	5	-0.007	0.007	0.003	0.003
		HIP	3.76	5	0.189	5	0.046	4	-0.032	0.032	0.028	0.001
	6-h	CTX	7	7	0.142	6	0.063	7	-0.032	0.032	0.019	0.010
		HIP	3.56	4	0.144	5	0.033	3	0.025	-0.025	0.003	0.012
Hprt	3-h	CTX	5.44	6	0.080	4	0.035	7	0.030	-0.030	0.000	0.001
		HIP	6.74	7	0.246	7	0.100	7	-0.046	0.046	0.031	0.059
	6-h	CTX	1.19	1	0.077	1	0.033	3	0.020	-0.020	0.002	0.001
		HIP	1.57	1	0.106	1	0.030	2	0.022	-0.022	0.005	0.005
Gapdh	3-h	CTX	1.68	1	0.041	1	0.032	4	-0.020	0.020	0.001	0.002
		HIP	3.46	4	0.166	4	0.040	3	-0.015	0.015	0.010	0.005
	6-h	CTX	2.51	3	0.099	3	0.032	2	0.023	-0.023	0.001	0.001
		HIP	2.34	3	0.106	1	0.019	1	0.039	-0.039	0.000	0.008
Pgk1	3-h	CTX	2.28	3	0.041	1	0.031	3	-0.019	0.019	0.001	0.003
		HIP	1.32	1	0.143	1	0.014	1	-0.015	0.015	0.002	0.000
	6-h	CTX	2.28	2	0.077	1	0.031	1	-0.001	0.001	0.008	0.002
		HIP	4.16	5	0.135	3	0.042	4	-0.012	0.012	0.016	0.007
Cypa	3-h	CTX	6	7	0.085	5	0.034	6	0.021	-0.021	0.002	0.003
		HIP	6.24	6	0.215	5	0.068	6	0.027	-0.027	0.056	0.004
	6-h	CTX	4.56	5	0.12	5	0.045	5	-0.025	0.025	0.003	0.009
		HIP	7	7	0.242	6	0.085	6	-0.070	0.070	0.002	0.138
Tuba4a	3-h	CTX	1.86	2	0.059	2	0.016	1	-0.003	0.003	0.000	0.000
		HIP	2.78	3	0.152	2	0.061	5	0.045	-0.045	0.010	0.024
	6-h	CTX	5.23	6	0.112	4	0.047	6	0.036	-0.036	0.004	0.006
		HIP	6	6	0.177	5	0.052	5	-0.008	0.008	0.043	0.002
Tbp	3-h	CTX	3.56	4	0.07	3	0.025	2	-0.003	0.003	0.005	0.000
		HIP	1.86	2	0.143	1	0.019	1	0.004	-0.004	0.012	0.000
	6-h	CTX	3.72	4	0.091	2	0.039	4	-0.020	0.020	0.002	0.005
		HIP	1.86	2	0.124	2	0.019	1	0.004	-0.004	0.003	0.001

Supplementary figures:
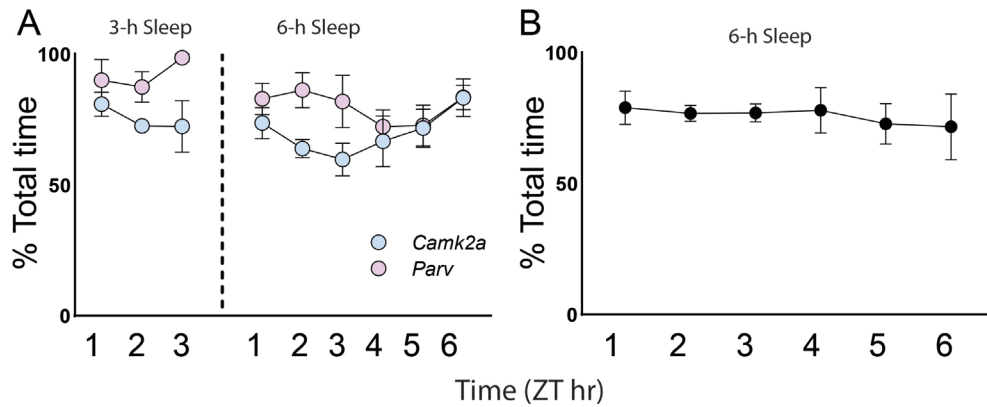
Figure S1. **Total sleep time in freely-sleeping mice.** Proportion of time spent in *ad lib* sleep between ZT0 and ZT3 or ZT6 for mice used in RT-qPCR experiments **(A)** and *in situ hybridization* experiments **(B)**. Sleep behavior (during which mice were observed to be inactive and in stereotyped sleep posture) was quantified in 5-min or 2-min intervals across the *ad lib* sleep period (for 6-h and 3-h experiments, respectively). Values expressed as a mean percentage of total time spent in sleep (± SEM), in 60-min intervals.
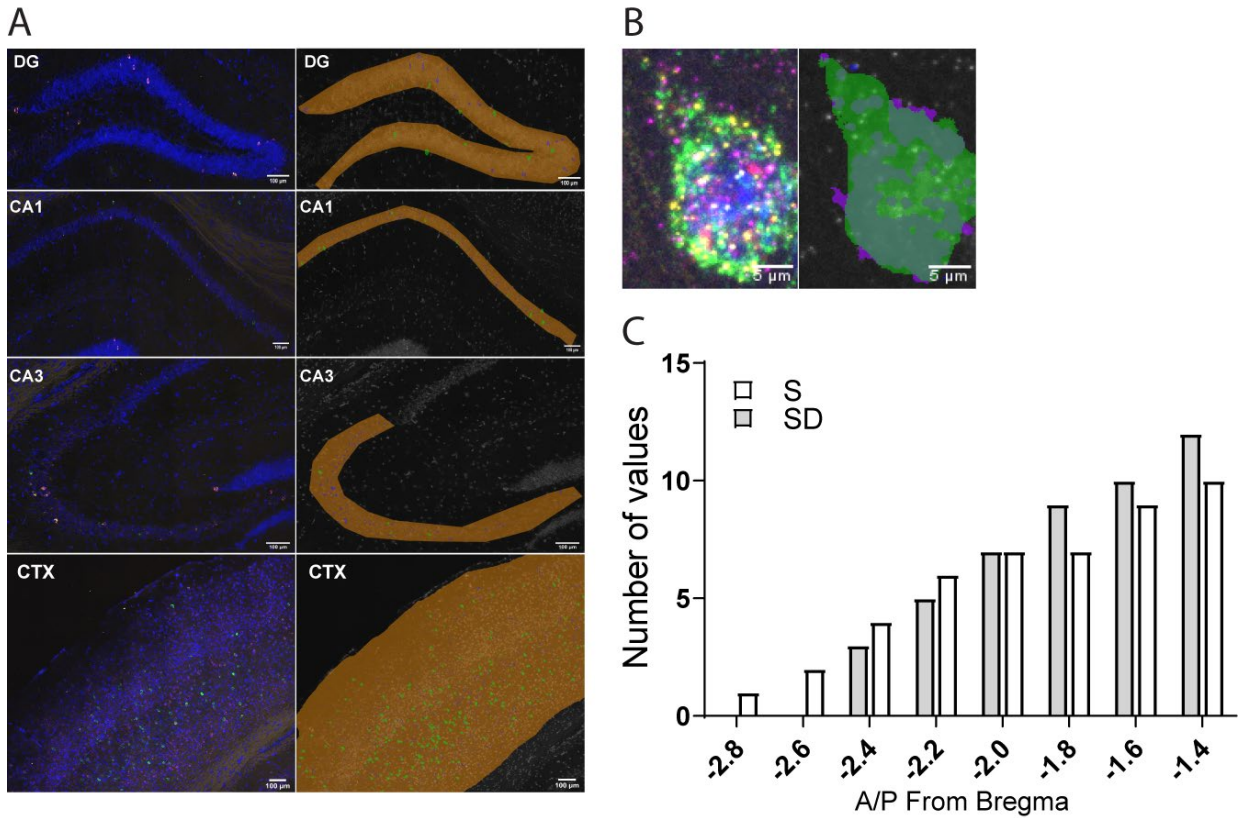
Figure S2. Strategy for quantification of fluorescence *in situ* signals. (A) Anatomical regions for quantification were demarcated manually (shown in orange). Within these anatomical regions, *Pvalb* (green) fluorescence delineated PV+ and non-PV+ ROIs. Background was defined as any area not expressing IEG (red) fluorescence. An automated protocol then calculated the total fluorescence intensity and area of each ROI and background area. These values were used to estimate the number of IEG fluorescence dots within each ROI. **(B)** Example of IEG and *Pvalb* fluorescence and identification. **(C)** Cumulative frequency distribution of A/P coordinates (relative to Bregma) for brain sections used in analysis.
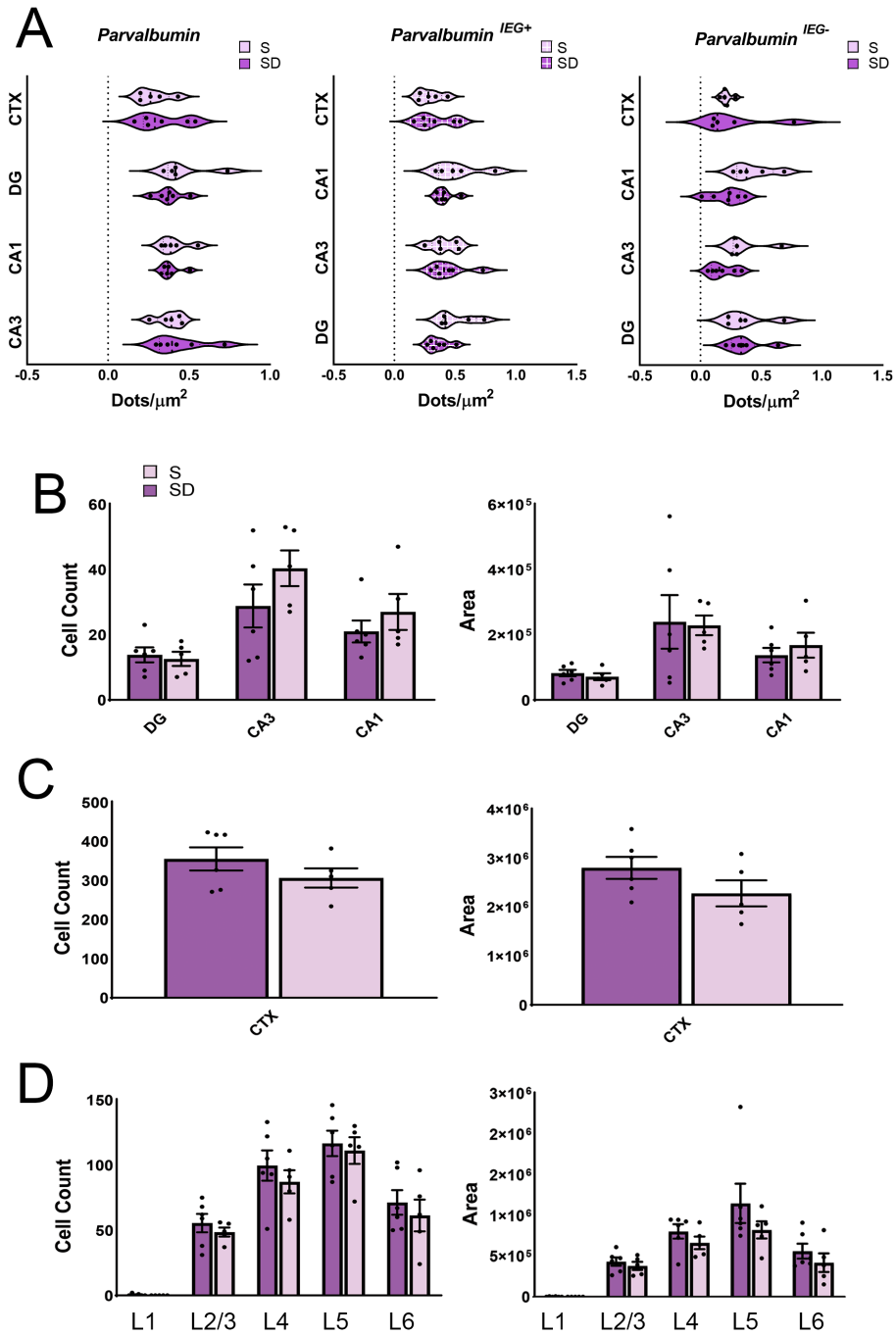
Figure S3. Mean *Pvalb* mRNA expression is similar between freely-sleeping and SD mice. **(A)** Mean *Pvalb* expression levels were similar in sleeping and SD mice, in all, IEG+, and IEG- PV+ interneurons. **(B-D)** Neither the total *Pvalb*+ cell count nor the *Pvalb*+ area differed between sleeping and SD mice, for either **(B)** hippocampal areas DG, CA1, or CA3, **(C)** whole neocortex, **(D)** or cortical layers 1-6. Values indicate mean ± SEM; *N.S.*, Holm–Sidak *post hoc* test vs. Sleep.

2.11 References:

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CHAPTER III

How Rhythms of the Sleeping Brain Tune Memory and Synaptic Plasticity

3.1 Abstract

Decades of neurobehavioral research has linked sleep-associated rhythms in various brain areas to improvements in cognitive performance. However, it remains unclear what synaptic changes might underlie sleep-dependent declarative memory consolidation and procedural task improvement, and why these same changes appear not to occur across a similar interval of wake. Here we describe recent research on how one specific feature of sleep - network rhythms characteristic of REM and NREM - could drive synaptic strengthening or weakening in specific brain circuits. We provide an overview of how these rhythms could affect synaptic plasticity individually and in concert. We also present an overarching hypothesis for how all network rhythms occurring across sleep could aid in encoding new information in brain circuits.

3.2 Introduction: Crescendo or diminuendo? The role of sleep in tuning synaptic strength in the brain

More than a century of study has made clear that sleep promotes both declarative memory storage and skill learning. Such processes are now known to be associated with

changes in the strength of connections between neurons in the brain - so-called "synaptic plasticity". Over the past two decades, evidence has accumulated that sleep directly promotes synaptic plasticity. This evidence suggests that sleep can have differing effects on synaptic strength, depending on the specific brain circuit under study and the animal's prior experience (**Figure 3.1**).

For example, proponents of the synaptic homeostasis hypothesis (SHY) cite a number of studies indicating that when animals remain awake for several hours, expression of a number of genes involved in synaptic plasticity (e.g., immediate early genes, neurotrophic factor genes) and synaptic localization of glutamatergic receptors are elevated in the neocortex [1]. When animals are allowed to sleep, levels of these indicators of synaptic strength decline. In support of the idea that glutamatergic synapses are strengthened during wake, firing rates in some neocortical neurons can also increase after a period of extended wake [2]. Recent microanatomical evidence (based on serial transmission electron microscopy) supports the idea that at least some neocortical synapses increase in size during a period of wake, relative to a period of sleep [3] (**Figure 3.1b, middle**). Taken together, these data support the idea that neocortical synapses undergo relative strengthening across wake, and relative weakening during sleep. However, a number of lines of evidence (some of which are outlined below) suggest that sleep effects on synaptic strength are more diverse and complex than the synaptic homeostasis hypothesis would predict.

First, recent data characterizing changes in individual neurons' firing rates over time show that sleep has heterogeneous effects within neural circuits (**Figure 3.1a**). Spontaneous firing rates of individual neurons in both cortex and hippocampus vary over several orders of magnitude, and multiple studies have found that across a period of sleep, higher-firing neurons undergo firing rate reductions, while sparsely-firing neurons show increases [4-6]. Critically, our recent experiments have shown that firing rate changes in both directions (i.e., increases and decreases) are blocked by a period of sleep loss [5].

In addition, a number of recent biochemical and anatomical studies indicate that in brain areas such as the hippocampus, sleep dependent shifts in plasticity related markers often diverge from what SHY would predict. For example, anatomical data in both area CA1 and DG show that sleep loss reduces (rather than increases) the number of dendritic spines in pyramidal neurons [7, 8]. Prolonged wake also leads to reduced cAMP levels in the hippocampus, which in turn disrupts the strengthening of glutamatergic synapses through long-term potentiation (LTP) [9]. Further, expression of the immediate-early gene Arc, which is required for LTP, long-term depression (LTD), and homeostatic plasticity, is simultaneously increased in neocortex *and decreased in hippocampal structures* following a period of extended wake [10].

Finally, available data suggests that in both hippocampus and neocortex, following a learning experience, synapses can be strengthened in a circuit-specific manner during sleep. In hippocampus, both neuronal firing rates and cellular indicators of synaptic strengthening increase during sleep in the hours following spatial or contextual

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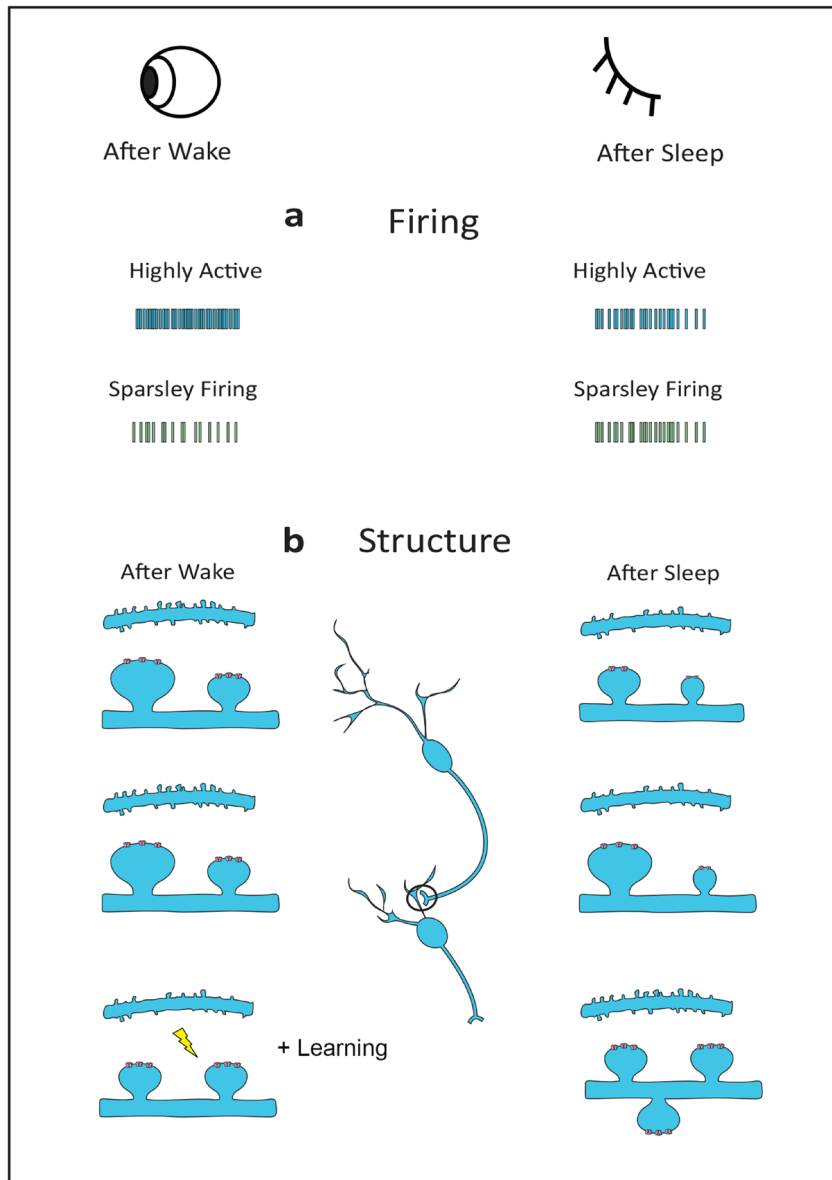
Figure 3.1: Overview of observed and hypothetical changes to neurons across sleep. Sleep-associated changes in firing rate (**a**) are heterogeneous, with highly active neurons (***top left***) undergoing sleep-dependent decreases in rate, and sparsely-firing neurons (***bottom left***) undergoing sleep-dependent firing rate increases. Changes in synaptic structure across sleep (**b**): as predicted by SHY (global synaptic downscaling; ***top row***), as seen in ultrastructural studies in non-learning animals (decreases in size of smaller boutons; ***middle row***), and as seen in cortical areas activated during prior learning (increases in synapse number and stability; ***bottom row***).

learning [11-17]. In the motor cortex of adult mice, dendritic spine growth occurs in a sleep-dependent manner immediately following motor learning [18] (**Figure 3.1b, bottom**). In the visual cortex of juvenile cats and adult mice, biochemical and electrophysiological indicators of synaptic strengthening are present during sleep in the hours following a novel visual experience [19-25].

An unanswered question is how plastic changes to brain circuitry come about during sleep. A plausible hypothesis (and one increasingly discussed among neuroscientists) is that the neuronal and network activity patterns that characterize sleep play a critical role in promoting specific types of synaptic plasticity [26]. Here, we review recent evidence that specific rhythms present in the mammalian brain (**Figure 3.2a**) during rapid eye movement (REM) and non-REM (NREM) sleep promote synaptic plasticity underlying the cognitive benefits of sleep.

3.3 A symphony of NREM sleep rhythms, and their role in synaptic plasticity: NREM sleep is a symphony of rhythms (**Figure 3.2a**). Some of these rhythms, such as slow wave activity (SWA; comprising delta [1-4Hz] and slow oscillation [< 1 Hz]), are continuous and present for prolonged periods throughout much of NREM sleep. Others, such as thalamocortical spindle (7-14 Hz) and hippocampal sharp wave ripple (> 100Hz) oscillations occur in discrete bursts at intervals, and are more prominent in particular phases of NREM. How these rhythms are coordinated with one another, and their function in processes such as memory consolidation, are areas of active investigation. Here we discuss how specific rhythmic patterns of NREM brain activity participate in regulating synaptic plasticity.
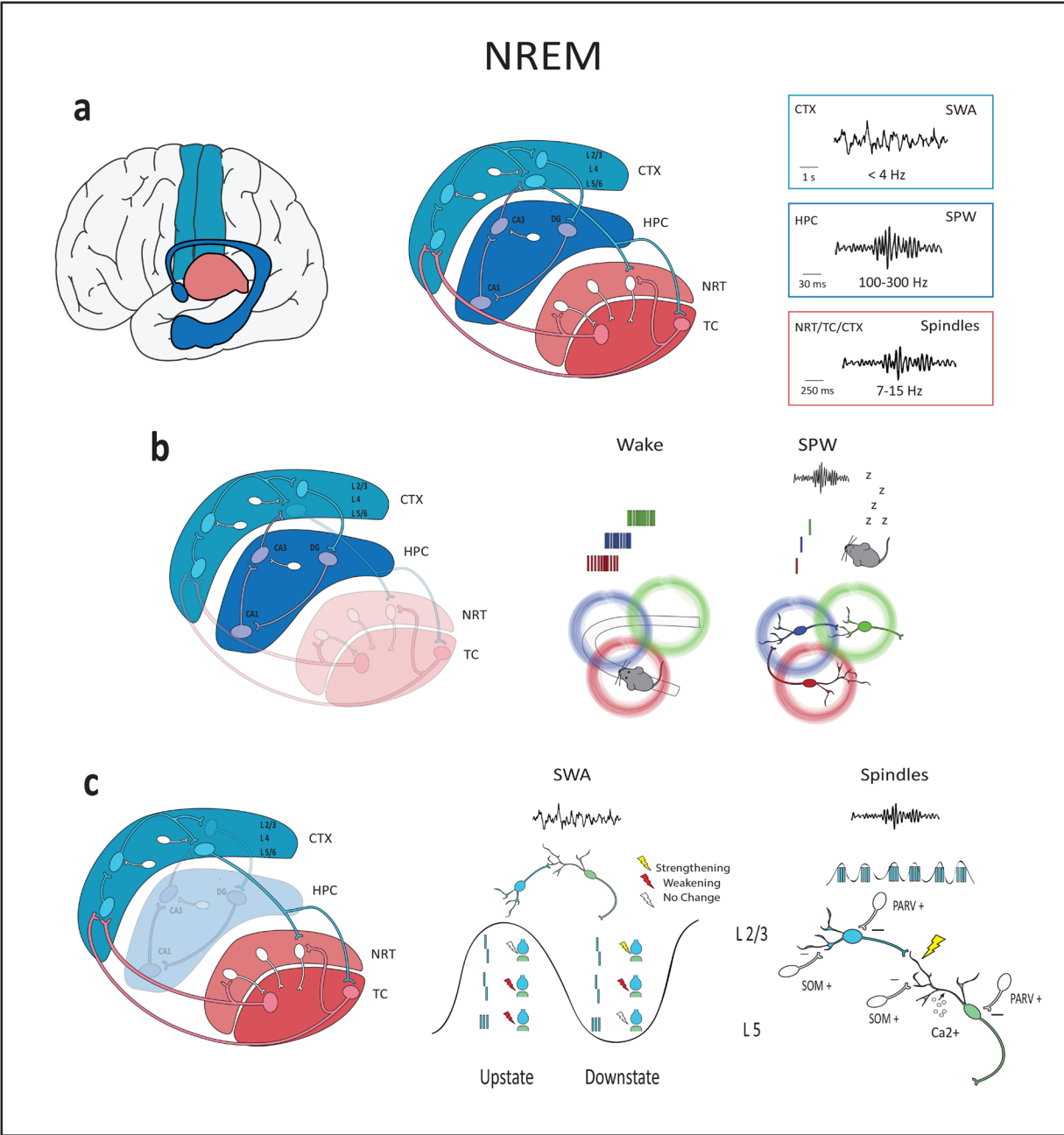
Figure 3.2: Rhythms of NREM sleep, and their effects on neural circuits. Sleep associated rhythms are expressed in memory-subserving hippocampal (indigo), cortical (cyan), and thalamic (red) circuits (**a, left and middle**). Various NREM-associated rhythms affect different subsets of these circuits (**a, right**). CTX =cortex; HPC = hippocampus; NRT = thalamic reticular nucleus; TC = thalamocortical relay nucleus. During NREM sleep SPWs, sequential replay of activity patterns among neurons activated sequentially during wake has been reported (**b**), in both hippocampal and cortical circuits. Additional NREM rhythm-specific mechanisms have been proposed to mediate plasticity in intracortical and thalamocortical circuits (**c**). These include selective depression and potentiation based on phasing of correlated neuronal activity during upstates and downstates of SWA in NREM (**c, middle**), intracortical changes in inhibitory interneuron activity during NREM spindles (**c, right**). SOM+ = somatostatin-expressing interneuron; PARV+ = parvalbumin-expressing interneuron; Ca2+ = calcium.

3.4 Adagio: Slow wave activity (SWA): Numerous studies published over the past two decades have linked one of the most prominent NREM sleep rhythms, SWA (used to refer to rhythms ≤ 4 Hz; **Figure 3.2a**), to consolidation of both declarative and procedural memories in human subjects. Often treated as a unified phenomenon, SWA is an amalgamation of two rhythms: a thalamically-generated delta (1-4 Hz) rhythm and the cortically-generated slow (< 1Hz) oscillation. Both total amounts and intensity (i.e., amplitude or temporal density) of SWA across a sleep interval have been positively correlated with some forms of declarative memory retention [27] or improvement on a wide range of procedural (e.g., sensorimotor) tasks [28-31]. An intriguing feature of SWA is that it in addition to being homeostatically regulated (i.e., augmented across the brain after a period of prolonged wake), it also appears to be regulated by brain activity during cognitive tasks in prior wake. Task-related increases in SWA in turn appear linked to task improvement [32]. For example, "local" SWA (where SWA is augmented over a particular region of cortex activated during prior waking experience) has received a great deal of attention, and appears to predict individuals' sleep-associated improvements on some tasks [29, 31, 33]. More recently, studies using non-invasive brain stimulation techniques have provided additional evidence for a causal role of SWA in promoting cognitive functions. Enhancing SWA in NREM (through auditory closed-loop stimulation or transcranial direct current stimulation [tDCS]) leads to improvements in declarative verbal memory [34-40]. Furthermore, it was demonstrated that SWA augmentation through tDCS can lead to functional (i.e. motor) improvements during recovery from stroke [41], and memory consolidation improvements in patients with mild cognitive impairment [42]. Conversely, disruption of SWA during NREM sleep can interfere with sleep-associated memory

consolidation [43]. Together these data suggest a causal role for SWA in promoting neurobiological events underlying cognition.

What are the neurobiological underpinnings of SWA's benefits for cognition? SWA displays numerous phenomenological features that are useful for consolidating plastic changes initiated in neural circuits during wake. Its homeostatic regulation means that slow wave intensity and amplitude are greatest immediately following wake, and particularly following prolonged wake. The local nature of this homeostasis means that areas highly active in wake (e.g. during learning) experience even greater subsequent SWA. Slow waves propagate across the cortex, often along the rostro-caudal axis [44, 45] an ideal scenario for plasticity between neighboring cortical regions, and possibly specifically in the regulation of top-down feedback circuits. Recent data suggest that temporal coordination of activity between adjacent cortical regions may play a critical role in promoting sleep-dependent memory processes. Miyamoto et al. [46] found that NREM-targeted optogenetic inhibition of top-down cortical feedback to primary somoatosensory cortex disrupted sleep-dependent consolidation on a texture discrimination task in mice. Moreover, rhythmically stimulating somatosensory and motor cortices synchronously (but not asynchronously) at a delta frequency (2 Hz) was sufficient to rescue memory consolidation deficits associated with sleep loss [46]. Finally (as we discuss in detail below), SWA coordinates other NREM rhythms to optimally synchronize neuronal activity across a number of brain circuits.

An unanswered question, and one that is vigorously debated in the field, is whether SWA promotes consolidation primarily via synaptic weakening, synaptic strengthening, or both [26]. Proponents of SHY have suggested that SWA in particular may lead to overall reductions in synaptic strength due to highly coincident spike timing between neurons, coupled with altered spike timing-dependent plasticity (STDP) rules that bias synapses toward depression during NREM sleep [47]. However, it is unclear whether invoking the precise alteration proposed in those studies is warranted. As reviewed elsewhere [26], available data suggest that NREM-associated changes in neuromodulation may limit STDP (either potentiation or depression) during SWA. In addition, STDP rules appear to vary substantially based on pre- and postsynaptic neurons' firing frequency and their pattern of firing (i.e., tonic vs. bursting)[48, 49]. There are limited physiological data addressing STDP rules during SWA (**Figure 3.2c**). Gonzalez-Rueda et al. optogenetically stimulated (presynaptic) layer 4 cortical neurons and carried out whole-cell current clamp recordings from (postsynaptic) layer 2-3 neurons in the barrel cortex during SWA in urethane anaesthetized mice. The authors found a general lack of STDP-based synaptic strengthening when pre- and postsynaptic neurons' firing was restricted to SWA up states. In contrast, conventional STDP (with both strengthening and weakening occurring depending on the order of pre- and postsynaptic neurons' firing) was present during down states [50]. The authors also found that, when restricted to up states, presynaptic stimulation alone elicited postsynaptic LTD. Another study carried out by Bartram et al. found weakening of synapses among layer 3 neurons in medial entorhinal cortex slices when subthreshold presynaptic inputs were paired with induced postsynaptic up states. However, this same study also found that during up states, presynaptic inputs paired with

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suprathreshold postsynaptic bursts of firing underwent LTP [51]. An important caveat is that, like many studies linking sleep to reduction in synaptic strength (reviewed in [26]), these two studies were carried out in juvenile (i.e., 2-3 week old) mice, where rates of synapse elimination are developmentally upregulated. An unresolved question is thus whether the same STDP rules are present in NREM up states later in life.

Several recent studies suggest that under some circumstances, coordinated firing between neurons during SWA induces synaptic potentiation. Optogenetic disruption of SWA during NREM is associated with impaired plasticity in the adult mouse visual cortex [24]; this form of plasticity is initiated by experience in prior wake, and is associated with synaptic potentiation [25, 52]. Similarly, a recent study found that evoked potentials in the cat cortex were enhanced selectively across periods of NREM sleep (but not across periods of wake or REM). They also found that presynaptic stimulation patterned to mimic that seen in SWA (but not that seen in wake) increased cortical neurons' excitatory postsynaptic potential (EPSP) amplitude [53]. Finally Kruskal et al. used *in vivo* imaging of calcium transients to identify neuronal ensembles activated in a stereotyped fashion and consistently recruited during SWA up states [54]. The authors found that within this ensemble, LTP was consistently induced by canonical STDP rules (i.e., following pre-before-post spike pairings). They also found that the amplitude of LTP reflected the level of neuronal activation over tens of ms prior to the pre-before-post firing, rather than at the moment of pairing. The same pairing protocol outside of the ensemble (and where prior neuronal activation was low) led to LTD [54]. Together, these studies suggests that subthreshold synaptic inputs to cortical neurons may induce synapse-specific weakening

during SWA up states, while suprathreshold inputs (i.e., those eliciting postsynaptic spiking) are simultaneously preserved or strengthened.

3.5 Messa di voce: Thalamocortical sleep spindles: Another characteristic feature of NREM sleep is the occurrence of discrete waxing-and-waning events (sleep spindles) comprised of 7-15 Hz rhythmic activity (**Figure 3.2b**). The occurrence of sleep spindles in NREM sleep have been extensively linked to cognitive function in human subjects [55]. A number of studies have found that post-encoding spindle density increases predict sleep-associated declarative memory consolidation [56, 57]. Pharmacological interventions that augment or decrease spindling activity in NREM have enhancing or disruptive effects on the consolidation process, respectively [58]. Sleep-associated improvements on a number of sensorimotor tasks are also predicted by task-associated increases in spindle density and amplitude [59-61]. Similar to reports for SWA, spindle activity appears to have topological specificity - increasing specifically over cortical areas previously involved in task acquisition during wake [62-64]. These task-associated increases in "local" spindling is predictive of sleep-dependent task improvement [65]. As is true for SWA, auditory or tDCS interventions that increase spindling following memory encoding or procedural task acquisition improves subsequent performance [35, 36, 38, 39, 42, 66].

What circuit-specific events might mediate the effects of spindling on cognitive performance? As is true for SWA, *in vivo*, spindles occur as traveling waves, where spindle up-states are present in physically adjacent regions of cortex in close temporal proximity. However, unlike slow wave oscillations which propagate along the rostrocaudal

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axis linking frontal, parietal, and occipital cortices, spindles instead tend to progress along a curving path connecting temporal, parietal, and frontal cortex, temporally linking activity between these regions [67]. In addition, available data from animal models suggests that spindle oscillations induce coherent phase-locked activity in thalamic and cortical neurons, which may be particularly important for sleep-associated plasticity in sensory and motor systems. For example, functional plasticity in neurons of the primary visual cortex (V1) of both developing cats [21] and adult mice [23] is correlated with the strength of phase-locking of individual neurons' firing to spindle oscillations. Both forms of plasticity involve augmentation of neuronal firing rate responses to visual input, and are mediated by the same intracellular pathways involved in LTP at cortical glutamatergic synapses [19, 25, 52]. In the latter system, orientation tuning of mouse V1 neurons is altered in a sleep-dependent manner following exposure to a visual stimulus of a specific orientation (orientation-specific response potentiation; OSRP)[23-25]. Available data suggest that potentiation of thalamic (i.e., LGN) input to V1 is an essential mediator of OSRP. Specifically, thalamocortical LTP induced between LGN and V1 occludes OSRP *in vivo*, and vice versa, suggesting a common underlying cellular mechanism [52]. Recent data from our lab show that OSRP is initiated during waking sensory experience, leading to orientation-selective response changes in LGN (but not V1) neurons. During subsequent NREM sleep spindles, coherence of activity between LGN neurons and V1 is augmented in an experience-dependent manner [24]. Coherence of V1 neurons' spiking with network activity during spindle oscillations predicts the extent of OSRP in individual mice [23]. Because OSRP is measurable across cortical layers in V1, this suggests that plastic

changes throughout the thalamocortical circuit could be driven by spindle frequency activity.

Recent experiments have aimed to test the causal role of sleep spindles in promoting synaptic plasticity. Hypnotic agents that tend to suppress spindle activity (while sparing other rhythms such as SWA) disrupt sleep-dependent V1 plasticity in developing cats, while those that preserve these rhythms support plasticity [68, 69]. Similarly, optogenetic manipulations that disrupt coherent firing during thalamocortical rhythms in NREM sleep disrupt sleep-dependent plasticity in the adult mouse visual system [24]. In support of the idea that entrainment of neuronal firing patterns by spindles drives synaptic plasticity, repetitive delivery of patterns of neuronal activity recorded from rat cortex during spindles *in vivo* are sufficient to drive LTP between cortical pyramidal neurons *in vitro* [70]. Pulsatile stimulation delivered to the cortex at a frequency consistent with spindle oscillations (10 Hz), but not at lower frequencies, can also evoke LTP *in vitro* [70] and enhanced sensory-evoked potential amplitudes *in vivo* [71].

How might spindle-frequency activity drive synaptic strengthening in cortical and thalamocortical circuits? Two recent findings suggest potential underlying mechanisms. An *in vivo* imaging study by Seibt et al (2017) recently described high levels of dendritic calcium influx (which occurred in a synchronized manner among neighboring cortical neurons) which was tightly linked to spindle frequency EEG activity [72]. Insofar as increasing dendritic calcium is a consistent correlate of (and indeed prerequisite for) LTP [73], this finding is completely consistent with other results linking spindle-frequency activity

to synaptic potentiation in thalamocortical circuits. Critically, this increase in dendritic calcium was not associated with a concomitant increase in neuronal spiking during spindle-rich sleep [72] - suggesting a non-Hebbian form of EPSP-driven plasticity, dependent on intra-EPSP timing [74, 75] during spindling. An explanation of the unique conditions found during spindles in the Seibt et al study (i.e., high dendritic calcium influx without an increase in somatic calcium or neuronal spiking) may be best explained by a second recent study using *in vivo* calcium imaging to study activity in specific cortical cell populations during NREM sleep. In that study, Niethard et al (2018) found that specifically in the context of spindle oscillations, somatostatin-positive (SOM+) interneurons (which target inhibition to cortical pyramidal neurons' dendrites) show decreases in activity, while parvalbumin-positive (PV+) fast-spiking interneurons (which target inhibition to pyramidal neurons' cell bodies) simultaneous show increased activity [76] (**Figure 3.2c**). The latter finding is intriguing in light of the finding that at least some forms of sleep-dependent cortical plasticity are preceded by cortical column-dependent changes in the activity level of fast-spiking interneurons [21]. Together these data suggest that a circuit-specific mechanism involving alterations in inhibitory neuronal populations could drive the augmented dendritic calcium influx seen in pyramidal neurons during spindling.

3.6 Molto vivace: Hippocampal sharp wave ripples (SWRs): A third network rhythm occurring characteristically during NREM sleep is the hippocampal sharp wave-ripple (SWR; **Figure 3.2b**). SWRs consist of large, synchronized-onset (i.e., sharp) waves of hippocampal network activity which initiate subsequent high frequency (100-300 Hz) oscillations, or ripples. Work carried out primarily in rodent models has shown that (as is

true for SWA and spindles) the frequency of occurrence and amplitude of SWRs in the hippocampus during NREM increases after a learning experience in wake [12, 77-79]. SWRs have also been observed in the temporal lobes of human subjects [80] where their frequency of occurrence has been linked to successful memory consolidation [81]. Critically, experimental models that disrupt SWR occurrence - either in genetic models of dementia [82, 83] or in animals with experimental disruption of hippocampal circuit activity during SWRs [12, 13, 84, 85] - memory consolidation deficits have been reported.

How might these rhythms support memory consolidation? NREM SWRs have received intensive study over the past two decades due to their capacity to induce temporally patterned activity in neuronal ensembles that reflect activity patterns in prior wake [86] (**Figure 3.2b**). This so-called "replay" has been proposed as a critical mechanism of memory consolidation [87]. Replay events in SWRs have been proposed to reflect sequential spatial information (i.e., transiting through "place fields" which activate specific hippocampal "place cells") [88], learned associations [89] and emotional valence [90], suggesting that these events reflect multiple facets of wake experiences. Available data suggests that replay events are linked to Hebbian plasticity mechanisms occurring during prior wake. O'Neil et al. found that pairs of hippocampal neurons whose place fields overlap more (and thus show more co-firing within 200 ms of one another) during exploration show greater co-firing during subsequent NREM SWRs [91]. As reviewed elsewhere [26], it is unclear whether sequential replay in the hippocampus (i.e. reflecting prior place field transiting in wake) is essential for circuit plasticity underlying sleep-dependent consolidation, or is simply a reflection of a well-established memory. Other

features of hippocampal network dynamics during post-training sleep (such as network stabilization, which also correlates with ripple occurrence) might be better indicators of plasticity during consolidation of new memories [11-13]. However, during SWR replay, large hippocampal ensembles are co-activated within compressed time frames that are ideal for STDP [92] and the high-frequency bursts occurring during SWRs mimic tetanic stimulation protocols used to induce hippocampal LTP [51, 93-95]. Thus it is reasonable to assume that SWRs can and do promote hippocampal circuit plasticity.

SWRs have also been implicated in information transfer from hippocampus to cortex during sleep (**Figure 3.2c**). Evidence in support of this idea has come from simultaneous recordings from the two structures, which demonstrated high levels of time-locked cortical neuronal activity triggered by NREM SWRs [92, 96]. Indeed, recent work suggests that SWR-associated hippocampal-cortical communication is a general feature within the association cortices [97] and sensory cortices [98]. There is also data to suggest that co-activation of the two structures could reflect prior experience. For example, reactivation of cortical neuronal ensembles co-activated during prior spatial task learning were triggered by SWRs [89]. Future studies will be needed to determine whether this SWR-linked communication induces plastic changes in circuits outside the hippocampus, and whether this it is necessary for sleep-dependent memory storage.

3.7 Polymeter: Nested NREM rhythms: A growing body of evidence suggests that SWA coordinates other oscillation to facilitate the cognitive benefits of sleep and underlying synaptic plasticity. Many human-subjects studies in which SWA is augmented through

non-invasive means found that generation of spindles in coordination with slower oscillations (i.e., at a specific and consistent phase relative to up-states) appears to be a consistent correlate of sleep-dependent task improvement [39, 66]. This suggests that SWA could act as a "carrier wave", coordinating the timing of other oscillations generated in interconnected circuits (e.g., spindles in thalamocortical circuits, SWRs in the hippocampus) throughout the brain. In support of this idea, dual-site recordings of the hippocampus and prefrontal cortex show that hippocampal sharp waves reliably emerge during the onset and offset of cortical SWA up states [99, 100]. Thalamocortical spindles are similarly locked to SWA up states [100], and rhythmic optogenetic activation of cortical neurons at frequencies associated with SWA leads to phase-locked spindle occurrence [24]. This results in fairly ordered temporal relationship of nested rhythms, wherein hippocampal SWRs occur in tight temporal coordination with spindles, with peak spindle- and ripple-frequency activity occurring simultaneously between cortex and hippocampus [100-102], and ripples occurring selectively in spindle troughs [101, 103, 104]. This temporal coordination of the three rhythms has been established in human subjects undergoing exploratory invasive recording [101, 105] as well as in rodents [98, 100, 106].

It's been hypothesized that the phase-amplitude coupling of these rhythms constitute an interregional dialogue between thalamocortical and hippocampal circuits during which correlated firing drives plasticity and information transfer between brain areas. The precise phase relationship of activity between interconnected brain regions may play an essential role in interregional communication and coordinated sequential replay of neuronal firing patterns coordinated between hippocampal and cortical

ensembles, resembling those occurring during prior experience. For example, Rothschild et al. recently found that neural activity patterns during NREM sleep in the rat auditory cortex predicted CA1 (hippocampal) SWR patterns, and vice versa, following learning of an auditory task [98]. A similar phenomenon has been reported to occur between rodent hippocampus and visual cortex during NREM sleep following visuo-spatial tasks [107]. This coordination appears to be a reliable correlate of sleep-dependent consolidation. A recent study demonstrated that successful training on a spatial object recognition task (i.e., one that led to consolidation of spatial memory across subsequent sleep) led to increased spindle-delta-ripple coupling during post-training NREM sleep. The authors also found that when brain stimulation triggering delta-spindle sequences was applied in coordination with SWRs (but not when it was applied out of phase with SWRs) consolidation of spatial memory across sleep improved significantly [108].

Taken together, recent data suggest that coordination of various circuit-specific (e.g., SWR activity generated in the hippocampus, spindling in the thalamus) oscillatory activities between brain regions may play a critical role in promoting brain plasticity. This coordination would not only optimize spike timing between brain areas, thus promoting STDP, it may also optimize information transfer between brain areas - a mechanism that has long been discussed with regard to attentional mechanisms during wake [109]. In other words, adaptive temporal coordination of activity among various oscillating circuits during NREM could provide a basis for *informative* plasticity between neurons in various brain structures, similar to plasticity driven by sensory experience in wake. While this coordination appears to be mediated by SWA, future studies will be needed to better

understand: 1) the precise circuit-level mechanisms involved, 2) how these mechanisms might be affected by learning and by sleep pressure, and 3) whether some of the functions attributed to SWA are mediated by its role in coupling rhythms across brain structures.

3.8 Cambiare: REM: Available data suggests that REM sleep is beneficial for a number of cognitive functions, including consolidation of episodic and emotional memory [110, 111], creative problem solving [112], perceptual learning [113, 114], and restoration of perceptual learning after interference [115]. However, the mechanisms through which REM could facilitate these processes are largely unknown. REM is associated with a variety of changes in brain physiology, including unique patterns of neuromodulation and network activity [26]. Recent findings suggest that various forms of synaptic plasticity occur throughout the brain during REM sleep. For example, following a novel experience during wake, expression of mRNAs [15], proteins [116], and phosphoproteins [22] associated with synaptic plasticity are upregulated in hippocampal and neocortical neurons during subsequent REM. Recent studies using continuous *in vivo* electrophysiological recording have shown that following sensory experience in wake that initiates cortical plasticity, neuronal firing rates are selectively augmented across bouts of REM in sensory cortex [5, 25]. This REM-associated increase selectively affects sparsely-firing neurons that encode sensory stimulus features more precisely; in contrast, high firing neurons with low feature selectivity show either no change or reduced activity across periods of REM [5]. One possibility is that the highly active neuronal population includes fast spiking interneurons - an inhibitory cortical neuron population exhibiting high spontaneous firing rates, and whose firing (compared with pyramidal cells) selectively declines across periods of sleep

[2, 21]. Thus the phenomenon of augmented firing in sparsely-active neurons across bouts of REM may be related to cell type-specific effects on neurons' overall activity, and/or changes in excitatory/inhibitory balance during REM sleep [117]. Long-term calcium imaging has demonstrated that, as is true during NREM spindles, during REM sleep, dendrite-targeting SOM+ interneurons show decreases in activity, while cell body-targeting PV+ interneurons' activity is augmented [118]. As described above, this alteration may provide an ideal circumstance for non-Hebbian synaptic potentiation (which may selectively affect lower-firing pyramidal neurons) during REM. It might also relate to a seemingly contradictory finding. Structural studies using longitudinal two-photon imaging to characterize layer 5 cortical dendritic spines following learning on a sensory motor task showed synaptic pruning during REM sleep (which selectively affected newly-formed dendritic spines) [119]. This may be explained if dendrite-targeted inhibition is significantly reduced during REM, leading to increased dendritic remodeling (e.g., strengthening of stronger and pruning of weaker synapses). Interestingly, and in contrast to studies of firing rate changes occurring across REM in the cortex, studies of firing in the hippocampus have shown that mean firing rates in that circuit are selectively decreased across REM bouts [120]. Thus one possibility is that REM may have circuit-specific effects on different brain areas.

Clearly, REM has the potential to alter synaptic strength, although debate about REM sleep's role in regulating synaptic strength is ongoing [117, 121, 122]. Resolving this issue will ultimately come down to a better understanding of causal mechanisms - i.e., what features unique to the REM sleep state contribute to specific types of plasticity. Recent

studies have approached this question by focusing on the role played by rhythms prominent in the brain during REM sleep (including the hippocampal theta rhythm and pontine-geniculate-occipital [PGO] waves) in promoting long-term memory formation.

3.9 Alla marcia: Hippocampal theta rhythm: The hippocampal theta rhythm (which depending on the study is either defined as a relatively broad [4-12 Hz] or narrow [e.g., 6-8 Hz] frequency band) is one of the most consistent and pronounced features of REM sleep (**Figure 3.3**). Driven by medial septal input to the hippocampus[123-126], this rhythm is mediated intrahippocampally by parvalbumin-expressing (PV+) fast spiking interneurons.[12, 13, 127]

While the relationship of hippocampal theta activity to waking functions (e.g., for encoding spatial information during navigation) has received extensive study, there is a growing body of evidence that REM theta may play a role in sleep-associated cognitive functions. Early reports from napping human subjects indicated that theta activity recorded over prefrontal cortex during REM was a predictor of emotional memory improvement [111]. Available data also suggest a link between REM theta and memory consolidation in studies using animal models. Early work suggested that the phase of hippocampal neurons' firing with respect to REM theta rhythms could be modified as a function of their activation during prior waking experience [128]. More recently, cued fear learning in rats was shown to increase theta coherence between hippocampus and amygdala during subsequent REM sleep, and this increase in coherence predicts the success of associative memory consolidation (i.e., increases in coherence are

proportional to offline associative gains) [129]. A growing body of data indicates that theta frequency activity is increased for several hours following single-trial contextual fear conditioning (CFC) in hippocampal area CA1, across both REM and NREM sleep [11-13], and that this increase predicts successful contextual fear memory (CFM) consolidation. Other studies have experimentally tested whether hippocampal theta can itself drive improvements in performance. An early study found that augmentation of theta activity (via medial septal stimulation) immediately following training on a spatial visual discrimination task improved rats' task performance 24 h later [130]. Consistent with this, a more recent study disrupting septal input to the hippocampus optogenetically in the hours following CFC led to a reduction in REM theta in CA1, and impaired CFM consolidation [123]. Two related studies inhibited hippocampal theta activity locally (within CA1), in the hours following CFC, leading to similar deficits in CFM consolidation [12, 13]. Conversely, optogenetically generating a theta rhythm in CA1 throughout a period of post-CFC sleep deprivation is sufficient to rescue CFM consolidation (which normally requires uninterrupted sleep) [13].

At the cellular level, why might REM theta (or hippocampal theta rhythms in general) support memory storage? Theta is known to support replay of hippocampal place cell sequences, following their sequential activation during experience [131] (**Figure 3.3**). Computational work suggests that: 1) the precise phasing of neuronal firing during replay can be modified as a function of learning-associated plasticity [132, 133], and 2) replay could emerge naturally from resonance with subsequent network oscillations [132]. Electrophysiological data suggests that CA1 pyramidal neurons resonate selectively at
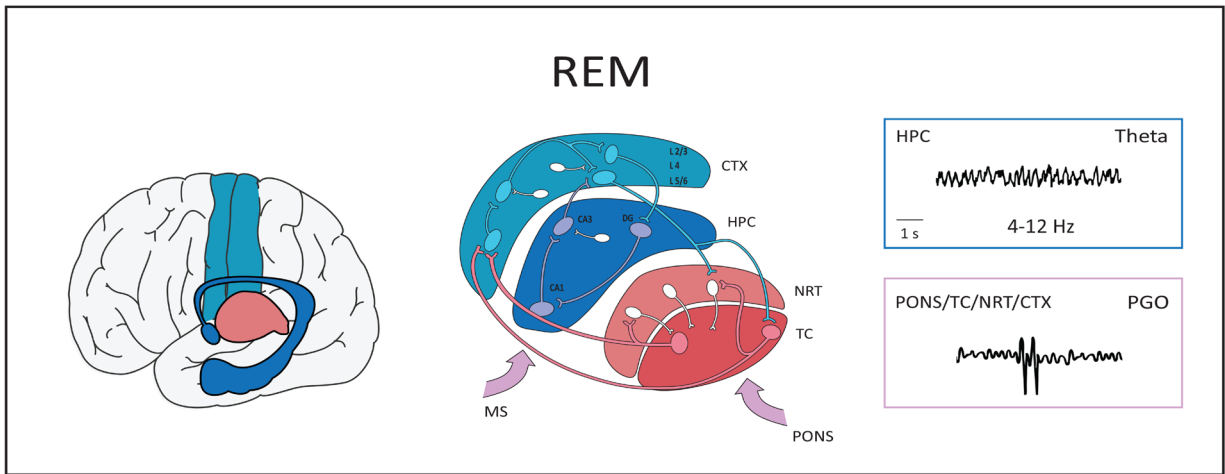
Figure 3.3 Rhythms of REM sleep. REM-associated rhythms (including REM sleep theta and PGO waves) are expressed in memory-subserving hippocampal (indigo), cortical (cyan), and thalamic (red) circuits and are modulated by input from the pons and medial septum (MS). CTX = cortex; HPC = hippocampus; NRT = thalamic reticular nucleus; TC = thalamocortical relay nucleus; MS = medial septum.

theta frequency, in response to rhythmic activity in parvalbumin-expressing (PV+) fast spiking interneurons [12]. This resonance is associated with increased consistency of spike timing relationships between neurons, an ideal scenario for driving STDP throughout the CA1 circuit [12, 13, 132]. Indeed, recent studies using CFM as a model of sleep-dependent memory consolidation have consistently found that long-term (i.e., over the time scale of several hours) stabilization of spike timing relationships among CA1 neurons is a robust predictor of consolidation [11-13]. Following CFC, pharmacogenetic or optogenetic disruption of theta rhythms leads to destabilization of CA1 spike timing relationships, and disruption of CFM consolidation [12, 13]. Optogenetic generation of theta oscillations increases the stability of CA1 spike timing relationships, and preserve memory in the face of sleep loss [12, 13]. Thus available evidence suggests that the highly regular theta-frequency activity that paces hippocampal neurons' firing during REM drives network plasticity, and plays a critical role for hippocampally-mediated memory consolidation.

3.10 Bravura: Pontine-geniculate-occipital (PGO) waves: PGO waves, like NREM spindles, occur as discrete biphasic wave events during sleep (as intermittent single waves during certain phases of NREM, and as repeating motifs throughout REM; **Figure 3.3**) [134, 135]. These high-waves of activity propagate (as the name implies) through the pons, the lateral geniculate nucleus of the thalamus, and the occipital cortex of species with highly-developed visual systems, and propagate from the pons to apparently communicate with numerous brain structures (and are thus simply called "P waves") in rodents [136]. A role of this wave in sleep-dependent memory consolidation (particularly for hippocampus- and amygdala-dependent memories) has been extensively investigated in

rodents. These studies have demonstrated an increase in P wave occurrence during REM following training on fear and avoidance memory tasks [137, 138]. Studies manipulating the generation of these waves following learning have demonstrated a clear causal role in promoting consolidation [139], which may be linked to P wave-dependent upregulation of kinase pathways and transcriptional activation of plasticity-mediating genes in target structures (such as the hippocampus and amygdala) in the hours following learning [14, 140, 141] (**Figure 3.3**). How these cellular events come about during and after PGO waves occurrence is an unanswered question, although it is clear that these waves can generate massive, highly synchronous depolarization among neurons in the circuits they propagate through. Thus it is not surprising that they tend to act as "carrier waves" that initiate other network rhythms (e.g., beta oscillations in the occipital cortex). In this respect, their behavior is very much analogous to sharp waves, which by virtue of generating synchronous depolarization, kick off ripple oscillations in CA1. During REM, PGO waves also occur with a specific phase relationship to theta rhythms occurring in the hippocampus [142]. Thus as is true in NREM sleep, in REM multiple rhythms harmonize activity across the brain. Such inter-regional synchrony, comprising rhythms of different cadences, may play a critical role in REM-associated plasticity and the cognitive benefits of REM sleep.

3.11 How the brain listens to sleep's symphony of rhythms - toward a unifying theory for sleep's role in synaptic plasticity: Numerous hypotheses have been proposed for the function of sleep-associated rhythms in mediating the cognitive benefits of sleep [26, 47, 86, 109, 136, 143]. And while oscillations at specific bands are clearly important

for plasticity across brain regions, we hypothesize that they can also influence synaptic plasticity in a broadly universal manner. We argue that during wake, new information is initially encoded in circuits based on experience-dependent changes in their firing rate (**Figure 3.4**). Rate coding (in which information is encoded based on how rapidly individually neurons fire) is frequently used by neurons during wake, e.g. to encode specific features of sensory stimuli. The idea that new information is encoded in the firing rates of specific neurons during experience has become widely accepted in recent years [144]. However, information encoding based on firing rate alone has several limitations. When neurons fire with a wide range of rates, plasticity based on STDP can be very ineffective. Furthermore, a purely rate-based readout should limit the contribution of sparsely active neurons, which provide important sensory information and are highly plastic [5], to an engram. Finally, the limited dynamic range over which individual neurons can vary their firing puts a limit on new information encoding in a purely rate-based system. For optimal information encoding and storage, non-rate-based information encoding strategies must be invoked.

We argue that sleep rhythms allow neurons to switch from a rate-coding mode, in which information is encoded based primarily on how rapidly each neuron in an ensemble fires, to a phase-coding mode, in which spike timing becomes critical for information storage [145]. We hypothesize that as the brain transitions from wake to sleep, it dynamically switches between rate and phase coding schemes (**Figure 3.4**). Sleep is well positioned to promote this switch for several reasons. The lack of incoming information to the brain during sleep leads to internal regulation of network dynamics. Changes in
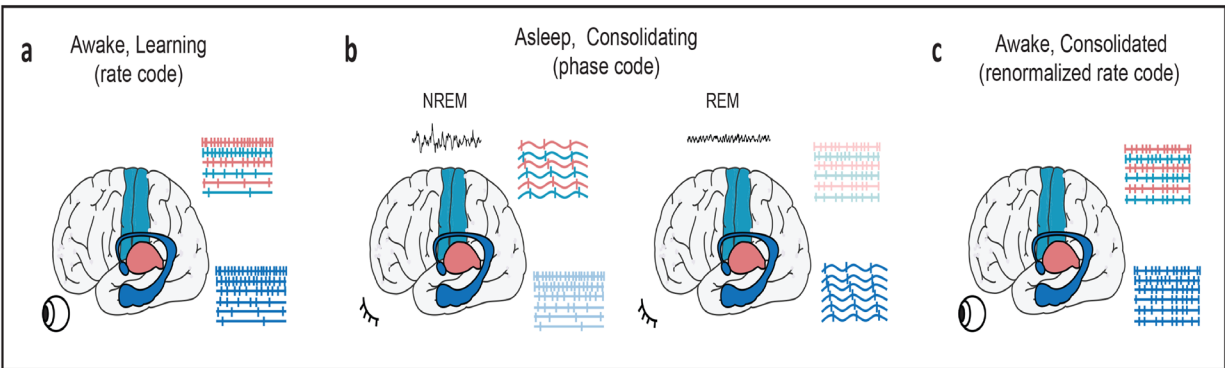
Figure 3.4 Resonance-based mechanisms for sleep-dependent plasticity. General mechanism for resonance-based plasticity. During wake, ensembles of neurons can encode new information, leading to selective changes in some neurons' firing rate and excitability (**a**). During subsequent network oscillations in sleep, resonance (wherein relative phasing is based on excitability) generates consistent spike-timing relationships between the neurons in the ensemble (**b**). Prominent rhythms present in thalamocortical networks during NREM sleep (**b, right**), and subsequently in the hippocampal network during REM sleep (**b, left**), can selectively bring these circuits into resonance based on circuit wiring and the intrinsic properties of circuit neurons. Spike-timing dependent plasticity among neurons in resonance leads to: (1) relative strengthening of synapses from highly activated neurons to the rest of the network, and (2) renormalization of firing rates (i.e. highly active neurons show reductions in rate, whereas sparsely firing neurons show increases, relative to prior wake). These changes are evident during subsequent wake (**c**).

neuromodulatory milieu simultaneously modifies the excitability of neurons, increasing their capacity for synchronization [146-148]. Finally, in many circuits, oscillatory neuronal activities during sleep drives the phase locking of neuronal activity across the network. This in turn leads to formation of stable spike timing relationships (the basis for phase coding and an ideal mechanism for STDP) across large networks.

Here we focus on one mechanism that relies on resonance, a biophysical property in which neurons have a heightened response to input of a specific frequency [149-151]. This mechanism alone can account for instructive plastic changes to network connections, based on prior experience. A neuron's resonant frequency shifts as a function of depolarization [152-154], meaning that neurons receiving larger synaptic inputs at higher rates, i.e. those which are more depolarized, will fire at an earlier phase. This general mechanism establishes a reliable input versus phase relationship across the network. During sleep-associated rhythms, neurons receiving more or larger excitatory synaptic inputs (based in part on prior experience during wake) will fire at earlier phases of a driving network oscillation (**Figure 3.3a**). This patterning can lead to the frequently-reported phenomenon of temporally-compressed replay of activity patterns occurring during prior wake [145]. Because during oscillations, these neurons will consistently fire prior to their postsynaptic partners, their excitatory inputs to other neurons (firing later in the rhythm, with a delayed phase) will be strengthened through STDP. Any reciprocal connections back to the phase-leading neuron will be weakened through STDP. Thus, when neurons within a network resonate with an oscillation, the input versus phase relationship paired with STDP will selectively strengthen connections from recently-activated "engram

neurons" to the rest of the network - an optimal scenario for systems memory consolidation.

A similar resonance-based mechanism would also act in the absence of prior learning. In this case, it would lead to differential phasing of highly active and sparsely firing neurons, with highly active cells leading. Assuming similar STDP-mediated effects, this would act to weaken inputs to neurons with initially high firing rates, and strengthen inputs to neurons with initially sparse firing. This phenomenon could underlie the rescaling of firing rates that appears to happen across neural circuits in a sleep-dependent manner [5]. This idea is similar to one recently put forth by Levenstein et al., who recently proposed NREM slow oscillations as a mechanism by which STDP rules could be optimized to promote heterogeneous firing rate changes among highly active and sparsely firing neurons in a network [6]. Critically, however, we argue that resonance is a general feature of neural circuits, not limited to slow oscillations. According to our model, any oscillation can drive synaptic changes so long as neurons resonate with its particular frequency band. Importantly, this resonance-based mechanism could account for instances of both synaptic weakening and synaptic strengthening across sleep, as well as more idiosyncratic phenomena such as forward and reverse replay [145].

For the reasons outlined above, we anticipate that the state-dependent switch between rate and phase coding would optimize information storage over widely-distributed, heterogeneous circuits across the brain. We predict that any rhythm (in any brain state) can drive synaptic changes so long as neurons' firing can resonate at its

frequency. The wiring of specific neural circuits, and neurons' intrinsic properties, will predispose them to resonance with oscillations of particular frequencies, which are naturally augmented during particular brain states (**Figure 3.4**). Thus, during NREM sleep, thalamic and cortical neurons will naturally resonate with high-amplitude SWA and spindle oscillations generated in thalamocortical circuits. During REM sleep, hippocampal neurons will resonate with regular, high-amplitude hippocampal circuit rhythms at theta frequency. Thus, it may be that the symphony of sleep-associated rhythms acts in concert, sharing a general mechanism by which they can weaken and strengthen synapses throughout the interconnected circuits of the brain.

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CHAPTER IV

Effects of NREM Slow Wave Activity on Neocortical Gene Expression and Visual Memory Consolidation

4.1 Abstract

Synaptic weakening across sleep has been hypothesized to be driven by slow wave activity (SWA) - 0.5-4 Hz rhythms prominent in thalamocortical activity during NREM sleep. Here, we used optogenetic manipulations in primary visual cortex (V1) to generate oscillations in the SWA frequency range, using cell type-specific expression of Channelrhodopsin2 (ChR2) in layer 6 corticothalamic (CT) neurons responsible for coordinating these oscillation *in vivo*. We assessed the ability of this manipulation to offset sleep deprivation (SD)-driven changes in neocortical gene expression and memory consolidation.

4.2 Introduction

The synaptic homeostasis hypothesis (SHY) posits that a fundamental sleep function lies in regulating synaptic strengths through net synaptic weakening. According to SHY, synapses undergo net potentiation during waking experiences which, if left unregulated, will lead to synaptic strength saturation, network instability, decreased signal-to-noise ratio, altered excitatory/inhibitory (E/I) balance, and a breakdown of

information processing. In response to waking potentiation, the hypothesis proposes that sleep re-normalize synaptic strengths through net synaptic weakening, restoring network E/I balance and preventing synaptic saturation. In support of SHY, multiple studies do show structural alterations to synapses, indicative of synaptic weakening, occurring preferentially after a period of sleep vs. sleep deprivation (SD). These include changes in presynaptic volume, spine density, and receptor composition (de Vivo et al., 2017; Vyazovskiy et al., 2008). Direct electrophysiological measures of neuronal excitability and synaptic strength likewise suggest that synapses experience sleep-dependent weakening (Vyazovskiy et al., 2009). And expression of immediate early genes (IEGs) implicated in synaptic plasticity mechanisms is increased with extended SD (Cirelli et al., 2004; Mackiewicz et al., 2007). Most recently, knockdown of the IEG *Homer1a* was shown to block sleep induced changes in synaptic composition (Diering et al., 2017).

However, these studies come with significant caveats. First, many were conducted with adolescent animals wherein the rate of synapse elimination is much higher than in adult brains (Havekes and Aton, 2020). Thus it remains unclear whether these findings reflect developmentally-regulated biological processes or an inherent function of sleep vs. SD. Second, effects of SD are often methodologically confounded by the use of novel objects or other forms of environmental enrichment. This makes it difficult to determine whether changes in physiology and gene expression are related to SD per se, or experiences during SD that are known to induce brain plasticity. Third, recent data suggests that different waking experiences - and accompanying neuronal activity patterns - may generate distinct transcriptional signatures across regions and cell types. This

complicates interpretation of IEG expression as a uniform measure of synaptic activity, and further complicates interpretation of studies using sensory enrichment for SD. Finally, and most critically, the degree to which these studies support SHY is limited by a lack of detail regarding underlying mechanisms for synaptic weakening.

SHY initially proposed that reduction in synaptic strength during sleep was a homeostatic (i.e. non-associative) plasticity mechanism ("downscaling") which responds globally to network perturbations. At the time of writing, the only example of *in vivo* homeostatic downscaling found to occur across sleep was found to be highly context dependent - occurring only after a period of monocular deprivation and not extending to both cerebral hemispheres (Torrado Pacheco et al., 2020). Firing rate changes across sleep are also not uniform, but instead differ as a function of baseline firing rates (Clawson et al., 2018; Miyawaki et al., 2019; Watson et al., 2016). And there are numerous examples of synaptic strengthening occurring during sleep (Puentes-Mestril and Aton, 2017). In response to these inconsistencies, an alternative causal mechanism for SHY mechanism was posited, in which sleep alters spike timing-dependent plasticity (STDP), an associated Hebbian mechanism, to bias synapses toward weakening (Hill and Tononi, 2005). Although supported by limited physiological data (Puentes-Mestril et al., 2019), there are again a number of ways in which this mechanism contradicts neurobiological data. Common between these proposed mechanisms is the critical role proposed for slow wave activity (SWA) in NREM sleep. NREM is characterized by low-frequency (0.5-4 Hz), high-amplitude network oscillations (SWA) in thalamocortical circuits. If wakefulness is prolonged, sleep pressure increases and SWA is increased in intensity (with higher-

amplitude waves). SHY posits that SWA mirrors sleep pressure because it is responsible for renormalizing synaptic strengths. Multiple studies suggest that NREM sleep plays an important role in memory consolidation (Aton et al., 2009; Ognjanovski et al., 2018; Puentes-Mestril and Aton, 2017; Puentes-Mestril et al., 2019).

Although intuitive and based on correlated features of sleep, these putative mechanisms for driving sleep-associated synaptic downscaling lack direct experimental support. To better characterize sleep dependent synaptic weakening mechanisms, and to test the SHY hypothesis, we sought to first manipulate SWA independent of behavioral state (using optogenetics) and characterize how it impacts gene expression correlates of sleep loss in the neocortex. We also used a new behavioral paradigm to demonstrate that sleep deprivation (SD) interferes with consolidation of visual recognition memory. Similar aspects of visual recognition (of familiar visual stimuli) have been linked mechanistically to a form of response plasticity initiated by prolonged visual stimulus presentation in primary visual cortex (V1), referred to as orientation-specific response potentiation (OSRP)(Cooke et al., 2015). This form of plasticity in V1 is dependent on post-stimulus sleep (Aton et al., 2014; Durkin and Aton, 2016) and coherent thalamocortical oscillations during NREM sleep between V1 and lateral geniculate nucleus (LGN) (Durkin et al., 2017). We also tested the effects of sleep vs. SD on consolidation of this form of memory, and also tested effects of optogenetic SWA manipulations on this process.

4.3 Materials and Methods

Mouse handling and husbandry

All animal husbandry and experimental procedures were approved by the University of Michigan Institutional Animal Care and Use Committee (PHS Animal Welfare Assurance number D16-00072 [A3114-01]). Animals were maintained on a 12:12h light/dark cycle (lights on at 9AM) with food and water provided *ad lib*. For all experiments, animals were individually housed (with beneficial enrichment) one week prior to the experiment and habituated by gentle handling five days prior to the experiment. Following habituation, and beginning at lights on, mice were either allowed *ad lib* sleep in their home cage or underwent SD by gentle handling. For freely-sleeping animals, sleep behavior was visually scored at 5-min intervals, based on criteria of immobility and stereotyped sleep posture, as used in prior studies (Delorme et al., 2019; Fisher et al., 2012; Pack et al., 2007).

Optogenetic stimulation of L6 CT neurons

For SWA manipulation experiments, *Tg(Ntsr1-cre)GN220Gsat* transgenic mice (*Ntsr1-Cre*; Gensat) were crossed to *B6;129S-Gt(ROSA)26Sor^{tm32(CAG-COP4*H134R/EYFP)Hze}/J* transgenic mice (ChR2-Cre; Jackson) to generate mice expressing channelrhodopsin 2 (ChR2) in layer 6 corticothalamic neurons(*Ntsr1::Chr2*). A second set of *Nrsr1-Cre* mice were virally transduced to express L6 ChR2 as described below. To assess the effects of CT stimulation on V1 neurons, *Ntsr1::Chr2* mice were anesthetized anesthetized with isoflurane (0.5-0.8%) and 1 mg/kg chlorprothixene (Sigma). A 32-site silicon probe with 250 μm spacing (Cambridge Neurotech) was slowly advanced into right

hemisphere LGN or V1 until stable recordings were obtained. And optical fiber was placed 0.5 mm below the cortical surface for delivery of laser light to V1 L6 neurons. A 15-min baseline was recorded, followed by 5-min periods of rhythmic optogenetic stimulation with 473 nm laser light (approximately 3 mW/mm^2; CrystaLaser) at the following frequencies: 0.5, 1, 2, 3, and 4 Hz. Stimulation periods were separated by 10-min intervals to allow neuronal firing to return to baseline levels. Following all optogenetic experiments, mice were perfused and brains were processed for histological assessment. Transgene exptic fiber and electrode position were validated prior to data analysis.

Surgical procedures

A subset of *Ntsr1-Cre* transgenic mice underwent bilateral viral transduction of V1 with an AAV vector to express either ChR2-GFP or YFP in a Cre-dependent manner, using previously-described methods (Durkin et al., 2017). For biochemical and behavioral optogenetic experiments *Ntsr1::Chr2* mice or AAV-transduced mice received bilateral implants of optical fibers, targeting V1. Mice were allowed 2-4 weeks of postoperative recovery prior to experimental procedures.

Quantitative real-time PCR (qPCR)

Following behavioral and optogenetic manipulations, mice were euthanized with an overdose of pentobarbital (Euthasol) and primary visual cortex (V1) was microdissected for mRNA isolation. For mice with implanted optical fibers, the area taken was approximately 1.5 mm in radius, with the center being the fiber site. Quantitative real-time PCR (qPCR) experiments were performed as previously described (Delorme et al.,

2019). mRNA was isolated using an RNeasy Micro kit (Qiagen). Total mRNA was quantified by spectrophotometry (Nanodrop Lite; Thermofisher) and diluted to equal concentrations of RNA. 20-500ng of total mRNA was used to synthesize cDNA using iScript's cDNA Synthesis Kit (Bio-Rad), cDNA diluted 1:10 in RNAse-free H_2O, and 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. Relative changes in gene expression were quantified using the ΔΔCT method.

Novel orientation recognition memory testing

Mice were trained for novel orientation recognition memory using a modified version of the task described in (Cooke et al., 2015). Briefly, following habituation to handling at ZT0, mice were placed in a 18" × 18" clear plexiglass arena, surrounded on all 4 sides by LED monitors. Mice were presented with a phase-reversing oriented grating (100% contrast, 0.05 cycles/deg, reversing at 1 Hz) of a single orientation (X°) on one of 4 monitors while the monitor opposite it displayed a solid gray screen. Stimulus presentation lasted 15 min after which they were returned to their home cage. 30-45 min later, mice were returned to the chamber and the same oriented grating-gray screen pairing was presented on the monitors opposite their first, for the same interval of time. Following training, mice were returned to their home cage for *ad lib* sleep or 6 h SD. A subset ChR2-expressing mice received bilateral optogenetic stimulation (3 Hz) throughout SD. On the second day of training, 12 hrs post-stimulus presentation, mice were alternately presented with gratings of the familiar X° orientation opposite a gray

screen and a novel X° + 90 orientation opposite a gray screen. Oriented grating-gray screen pairings were presented on monitors opposite their firsts during subsequent presentations for a total of two presentations for each oriented grating. Interaction with the two oriented gratings was quantified as time spent actively moving within the stimulus zone (portion of arena with x°or x°+90oriented grating) relative to the grey screen zone.

4.4 Mice constitutively expressing ChR2 show sleep-like decreases in plasticity-related gene expression after optogenetic generation of SWA

To test the role of SWA in regulating state-dependent neocortical gene expression, we expressed channelrhodopsin2 (ChR2) in a Cre-dependent manner in layer 6 corticothalamic (CT) neurons in *Ntsr1-Cre* mice (**Figure 4.1**). We used a range of stimulation frequencies (0.5-4 Hz) previously shown to augment SWS oscillations in the delta (0.5-4 Hz) and spindle (7-15 Hz) frequency bands (Durkin et al., 2017). ChR2 expression was achieved either constitutively (by breeding to Chr2 transgenic mice) or via AAV transduction of V1 bilaterally. For comparison of effects of SWA with natural *ad lib* sleep vs. SD, two groups of mice underwent no optogenetic manipulations, but were either allowed *ad lib* sleep (n = 5) or underwent 6-h SD by gentle handling (n = 5) , starting at lights on (ZT0). Two additional groups of mice underwent SD, with bilateral light delivery to V1 at either 1 Hz (n = 5) or 3 Hz (n = 5) throughout the SD period. Immediately following these manipulations, the neocortex (primary visual cortex; V1) was microdissected for qPCR quantification of mRNA abundance (**Figure 4.1a**).
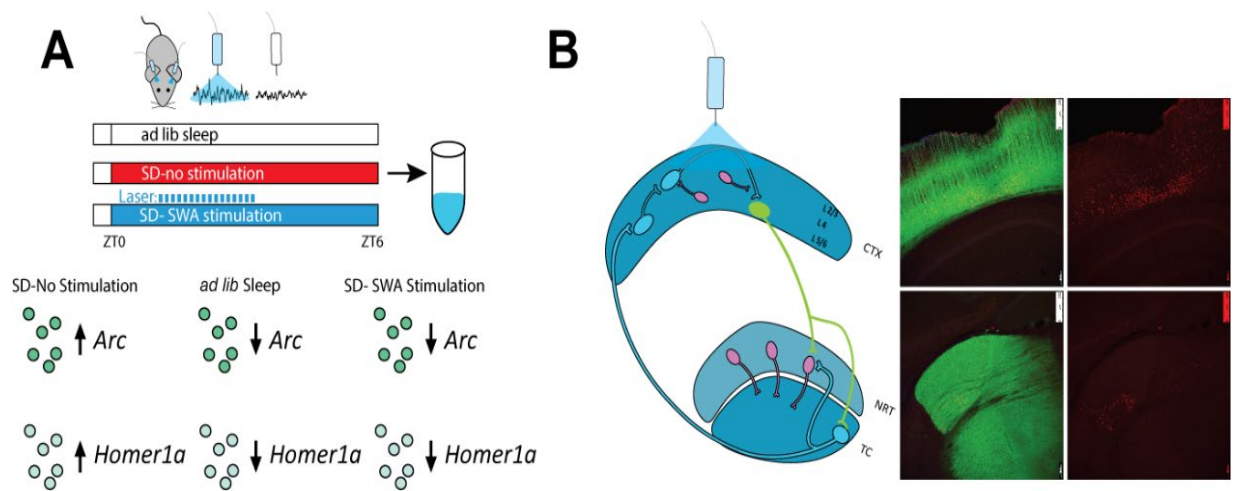
Figure 4.1. Experimental design. (**A**) Transgenic mice expressing ChR2 in layer 6 CT neurons were either allowed ad lib sleep starting at ZT0 (lights on), were sleep deprived (SD) for 6 h starting at ZT0, or were sleep deprived with rhythmic optogenetic stimulation of V1 layer 6 neurons. Mice were kept awake with gentle handling and light tapping to the cage and optogenetic fibres were coated in black paint to avoid excessive visual stimulation. Following these manipulations, neocortex containing V1 was microdissected for mRNA extraction and qPCR quantification of transcript abundance. Predicted changes for IEGs *Homer1a* and *Arc* are shown. (**B**) Expression of cFos in V1 and LGN following rhythmic stimulation of layer 6 neurons.

As shown in **Figure 4.2**, SD led to significant increases in V1 expression of *Homer1a* and *Arc* transcripts relative to *ad lib* sleep ($p < 0.01$ and 0.05, respectively, Holm-Sidak *post hoc* test vs. sleep). Both 1 Hz and 3 Hz stimulation of V1 throughout SD reduced expression back to levels seen in sleeping mice (**Figure 4.2**) ($p < 0.05$, Holm-Sidak *post hoc* test vs. SD).

4.5 Virally-transduced mice show no changes in gene expression associated with optogenetic generation of SWA

A second cohort of ChR2-expressing or (as a negative control) YFP-expressing mice (with V1 transduced virally) underwent the similar behavioral procedures. Mice expressing both transgenes underwent SD with rhythmic light delivery to V1 bilaterally. For the first experiments we used the lowest frequency in the SWA range, 0.5 Hz, for optogenetic stimulation. As shown in **Figure 4.3**, ChR2-transduced mice did not show the same decrease (relative to YFP-expressing mice) in *Arc* and *Homer1a* expression in V1 during SD (**Figure 4.3A**, *N.S.*, Holm-Sidak *post hoc* test). One possibility is that ChR2 expression itself was variable due to differential AAV-mediated transduction in V1, leading to variable changes in SWA. However, the level of expression for *Arc* and *Homer1a* transcripts was not significantly correlated with the amount of *Chr2* mRNA expressed in the tissue (**Figure 4.3C**, *N.S.* Pearson correlation). To further address this issue, we compared the effects of rhythmic light delivery at different frequencies on V1 network (local field potential; LFP) activity in mice expressing ChR2 via viral transduction or constitutively (**Figure 4.4A**). We found that optogenetically-driven changes in SWA measured by LFP power spectral densities (PSDs) were generally more modest in virally transduced mice than constitutively-expressing mice (**Figure 4.4B-C**), and were only

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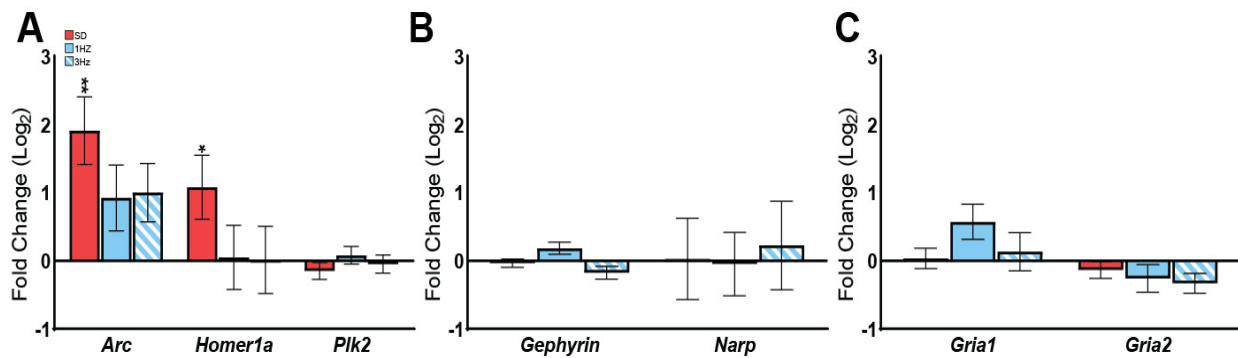
Figure 4.2. Optogenetic stimulation of neocortex at SWA frequencies offsets SD-driven changes in gene expression. V1 transcripts known to be upregulated in neocortex during SD and involved in synaptic plasticity were quantified using qPCR. Values indicate mean ± SEM for fold change vs. sleep for SD + no stim (red), SD + 1 Hz stim (solid blue) and SD + 3 Hz stim (hatched blue). All statistical tests were done on ΔCT values. Values indicate mean ± SEM with propagated error from ad lib sleep and SD groups. *, and ** indicate $p < 0.05$ and 0.01 vs. values for sleeping mice, respectively, Holm-Sidak *post hoc* test.
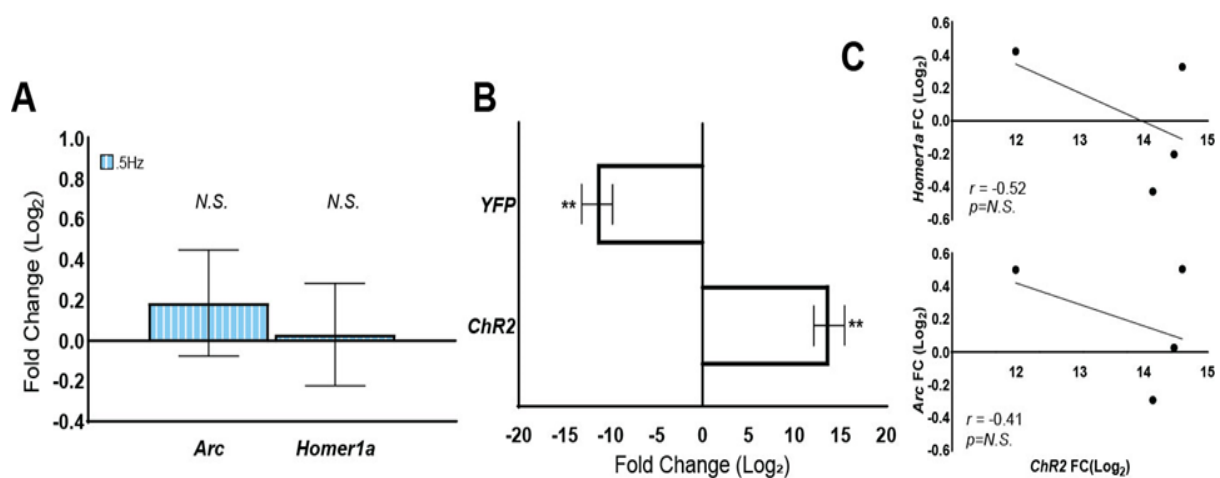
Figure 4.3. Optogenetic stimulation of neocortex at 0.5 Hz does not alter SD-driven changes in gene expression in virally-transduced mice. (**A**) qPCR-based quantification of V1 *Arc* and *Homer1a* transcripts in V1 of SD mice. Values are expressed as fold change from mean values from YFP-expressing mice. (**B**) Relative difference in Chr2 transcript abundance between YFP- and ChR2-transduced mice (**C**) Transcript levels for *Arc* and *Homer1a* did not correlate with expression of *Chr2* within V1.*r* and *p* values from Pearson correlation shown in panels. All statistical tests on qPCR data were done on ΔCT values. Values indicate mean ± SEM with propagated error from ad lib sleep and SD groups. *N.S.* indicate $p > 0.05$ vs. values for sleeping mice, respectively, Holm-Sidak *post hoc* test.
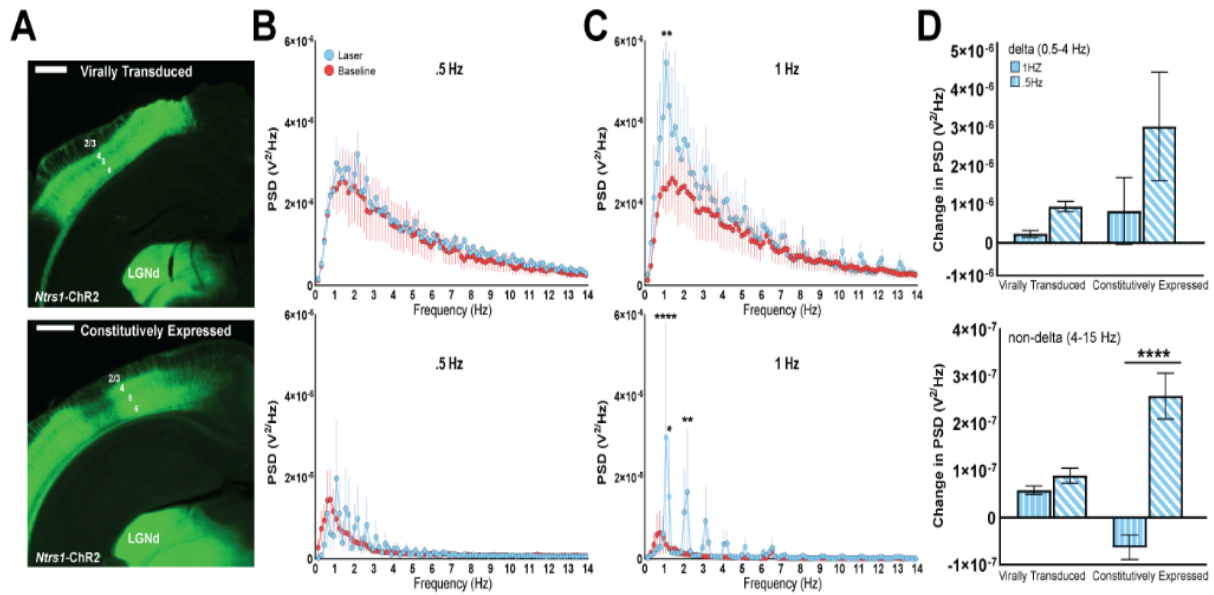
Figure 4.4. Effects of rhythmic light delivery at SWA frequencies in virally-transduced and constitutively-expressing mice. (**A**) Transgene reporter expression for ChR2 in a virally-transduced (**top**) and constitutively-expressing (**bottom**) Ntsr1-Cre mice. (**B-C**) power spectral density (PSD) in V1 at baseline (red) and during rhythmic light delivery to V1 at 0.5 (**B**) and 1 (**C**) Hz. (**D**) Mean (±SEM) PSD changes during optogenetic stimulation at 0.5 and 1 Hz (from baseline) within SWA (**top**) and theta/spindle (**bottom**) frequency bands for virally-transduced vs. constitutively-expressing mice. ** and **** indicate $p < 0.01$ and $p < 0.0001$, Holm-Sidak *post hoc* test.

significant during 1 Hz stimulation (**Figure 4.4C-D**). Constitutively-expressing ChR2 transgenic mice also showed significant increases in spectral power at higher frequencies (in the theta and spindle frequency range, outside of the SWA band).

We conclude from this that differences in gene expression between the first and second cohort could be due to either mode of transduction of ChR2 or to frequency of optogenetic stimulation (0.5 vs. 4 Hz). To test this, a third cohort of virally-transduced mice expressing ChR2 underwent SD with 1 Hz light delivery (**Figure 4.5**). For these mice, SD-driven changes in the expression in V1 of Homer1a and Arc were similar to those seen in SD mice without optogenetic stimulation. Thus differences seen in SD-driven gene expression in constitutively-expressing ChR2 transgenic mice may be due to the mode of transduction, where expression among layer 6 CT neurons is more widespread.

4.6 Optogenetic mimicry of SWA alters visual recognition memory

Because results of qPCR-based studies were somewhat inconclusive, we next assessed effects of sleep, SD, and optogenetically-driven SWA mimicry on a recently-reported form of visual memory (Cooke et al., 2015) (**Figure 4.6**). To do so we used a variation of the novel object recognition task that uses familiar and novel orientation gratings in lieu of physical objects. In adult mice, brief exposure to phase-reversing, oriented gratings results in enhanced cortical (V1) responses to stimuli of the same orientation. Our lab has shown that changes in the visual cortex related to this form of

Figure 4.5. Optogenetic stimulation of neocortex at 1 Hz does not alter SD-driven changes in gene expression in virally-transduced mice. V1 transcripts for *Homer1a* and *Arc* were similarly increased after SD, regardless of optogenetic stimulation at 1 Hz, in virally-transduced mice. All statistical tests were done on ΔCT values. Values indicate mean ± SEM with propagated error from *ad lib* sleep and SD groups. *N.S.* indicate p > .05 vs. values for sleeping mice, respectively, Holm-Sidak *post hoc* test.
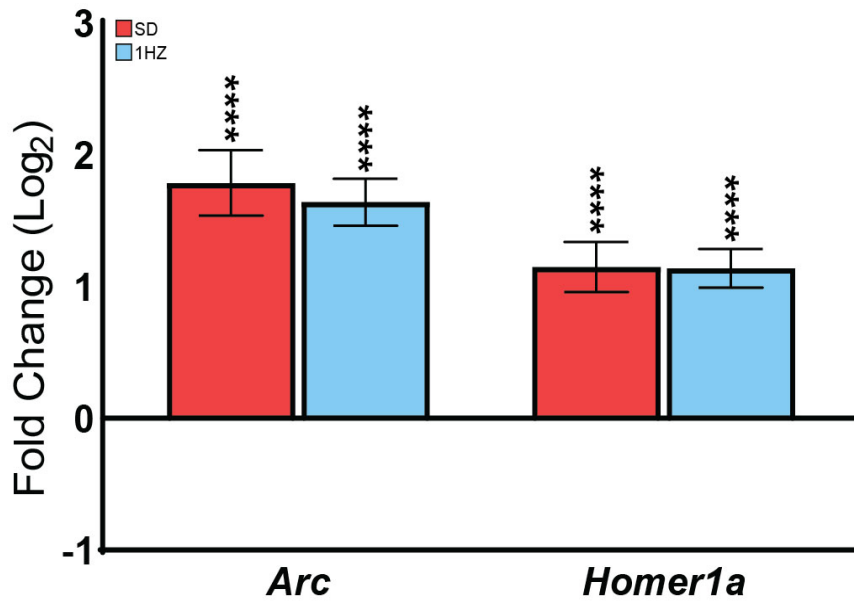
learning is disrupted either by SD or by optogenetic attenuation of SWA during SWS (Durkin et al. 2017). Work has also shown that this form of learning is dependent on the same cellular mechanisms underlying long term potentiation (LTP) and can be precluded via application of an inhibitor peptide in V1, suggesting information storage(Cooke et al., 2015). In using our modified task, we are then able to interrogate the role of SWA in a form visual memory dependent on both sleep and synaptic plasticity. We first examined the role of sleep in consolidating this memory, using C57Bl/6J mice (**Figure 4.7A**). We found that after wild type mice were presented with an X° oriented grating at lights on the first day, interaction with repeated presentation of X° (familiar) 1 Hz phase-reversing oriented gratings the following day was significantly reduced compared with interaction with an X+90° (novel) grating. This discrimination between novel and familiar oriented gratings was eliminated in mice that underwent 6-h SD following initial presentation of the X° grating. These experiments were repeated in *Ntsr1::ChR2* mice, where sleep and SD groups were compared with SD + 3 Hz mice, which had 3 Hz light delivery to V1 across the SD period (**Figure 4.7B**). While no significant differences were found between sleeping and SD *Ntsr1::ChR2* mice, SD + 3 Hz stimulated mice discriminated between familiar and novel gratings in a manner similar to freely-sleeping wild-type mice.

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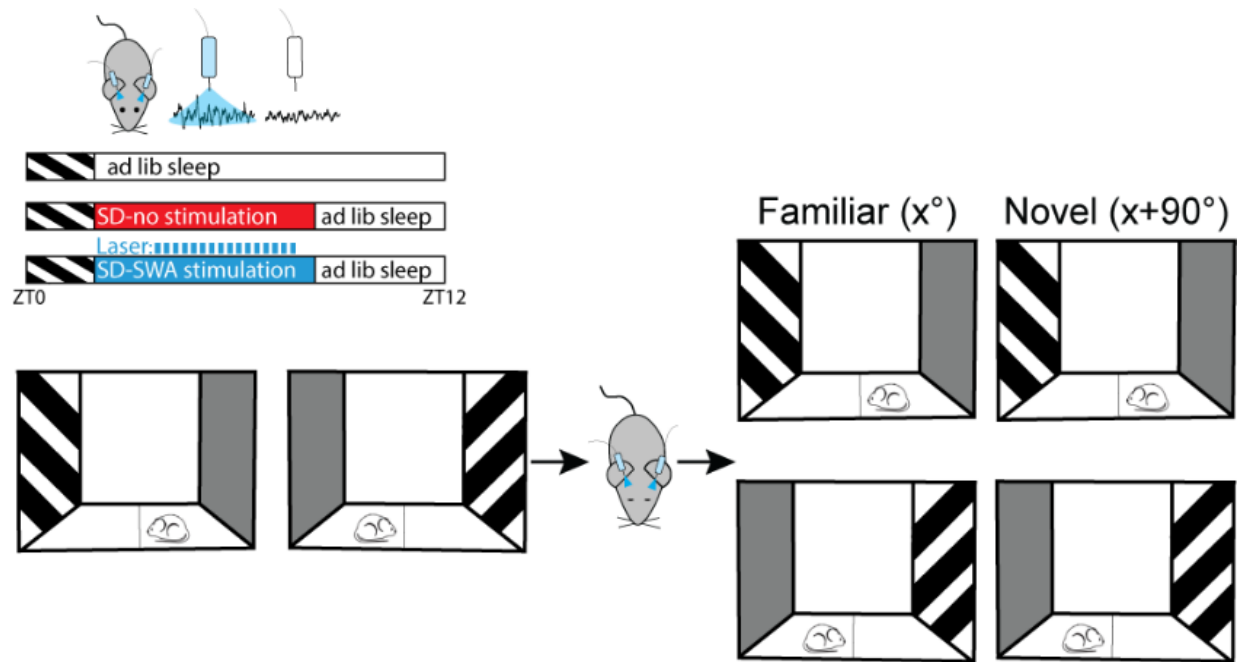
Figure 4.6. Experimental design for testing effects of SD and SWA on visual recognition memory. Mice were trained by repeated presentation of an X° oriented grating and a grey screen opposite the grating at ZT0. Unused monitors displayed a solid black screen throughout stimulus presentation. Following training, mice were either allowed *ad lib* sleep, or underwent 6-h SD, in their home cage. A subset of *Ntsr1::ChR2* mice received rhythmic light delivery to V1 during SD. 12 h after training, mice were re-presented with the familiar X° grating and a novel X+90° grating opposite their respective grey screens to quantify differences in interaction based on recognition of the familiar stimulus (preference). Preference was quantified as difference in time spent interacting with an oriented grating and its opposite grey screen over total interaction time.
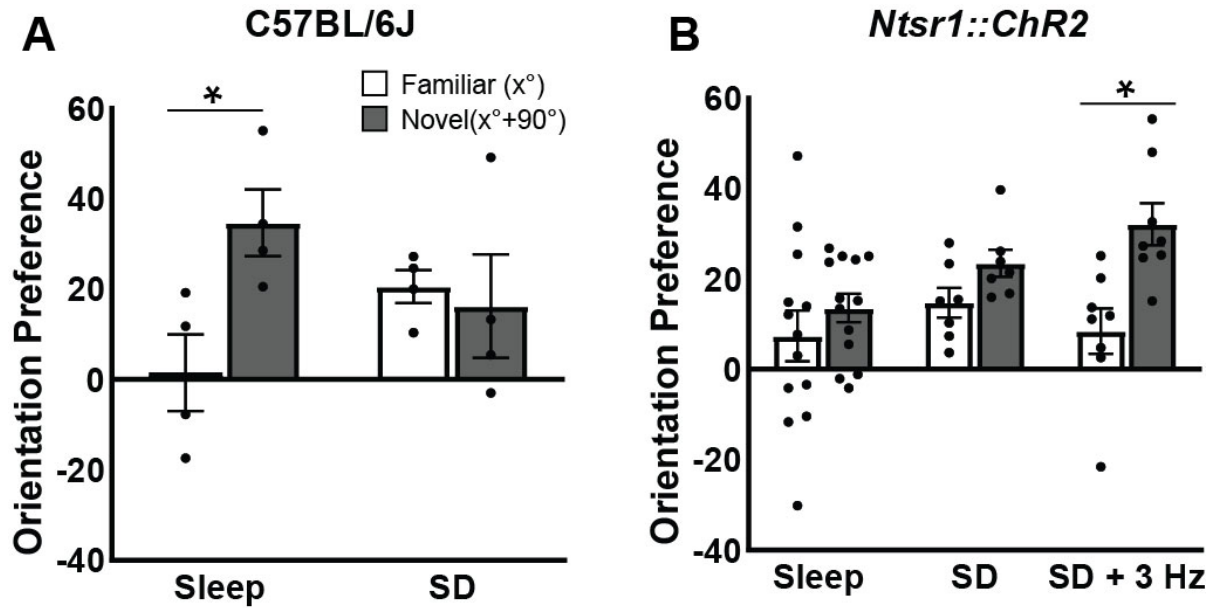
Figure 4.7. Effects of SD and SWA-frequency optogenetic stimulation of V1 on visual recognition memory. (**A**) C57BL/6J mice show evidence of recognition of the familiar (X°) oriented grating after *ad lib* sleep, but not after SD. (**B**) While neither sleeping nor sleep deprived *Ntsr1::ChR2* mice showed significant differences in interaction with familiar (X°) or novel (X+90°) oriented gratings, SD with rhythmic (3 Hz) light delivery to V1 during SD increased recognition memory. Symbols indicate orientation preference of individual mice. * indicates $p < 0.05$, Mann-Whitney U test.

4.7 Discussion

Using optogenetic-driven mimicry of SWA, we show that the simulation of slow wave oscillations in SD mice can preclude IEG correlates of sleep loss and rescue visual memory consolidation. Studies looking to clarify the role of SWA in sleep dependent weakening have often found divergent, if not conflicting, mechanisms that differ in critical ways (heterosynaptic vs Hebbian), while failing to account for many of the observed phenomena implicated in sleep dependent weakening (Bartram et al., 2017; Czarnecki et al., 2007; Gonzalez-Rueda et al., 2018; Peigeat et al., 2015; Puentes-Mestril and Aton, 2017). Here, we've focused on establishing whether there exists any causal relationship between SWA and state dependent gene expression. We find that rhythmic stimulation of layer 6 corticothalamic neurons at SWA frequencies (1 Hz and 3 Hz) blocks SD-driven increases in Arc and Homer1a gene expression (**Figure 4.2**). Paradoxically, optogenetic stimulation induces robust cFos expression in V1 and the LGN (**Figure 4.1**, not quantified). Given that cFos regulates the transcription of other IEGs, we might expect significant increases in *Arc* and *Homer1a* to follow (Hu et al., 2010). Our results show otherwise, suggesting that SWA mimicry may selectively alter gene expression. *Arc* and *Homer1a* significantly correlate with sleep loss (Delorme et al., 2019; Maret et al., 2007) and are critical in the expression homeostatic synaptic plasticity (Hu et al., 2010; Shepherd et al., 2006; Siddoway et al., 2014) and sleep homeostasis (Ahnaou et al., 2015; Suzuki et al., 2020). And, as previously mentioned, knockdown of *Homer1a* was most recently shown to block sleep induced changes in synaptic composition (Diering et al., 2017). The more modest increase in expression in our SWA mimicry group (**Figure 4.2**) suggests that our stimulation protocol either disrupted homeostatic responses to

sleep loss or was able to mimic the effects of sleep (i.e. induce weakening). Although prior studies have shown that rhythmic stimulation of neurons at 1-3 Hz can induce homeostatic downscaling(Goold & Nicoll, 2010) in excitatory cells, until changes in glutamatergic receptor expression and firing activity can be assayed, we cannot say whether our mimicry blocked sleep homeostatic or weakened cortical networks (Vyazovskiy et al., 2008; Vyazovskiy et al., 2009).Critically, we were unable to replicate this effect in AAV-transduced mice, finding no discernable change in gene expression at 0.5 Hz (relative to stimulated YFP-expressing mice) or 1 Hz (relative to non-stimulated ChR2-expressing mice). This may stem from two critical differences in our cohorts. First, our initial *Ntsr1::ChR2* cohort constitutively expressed ChR2 in all regions of the neocortex at high levels (not quantified) whereas our AAV-transduced cohorts showed more localized expression (not quantified, **Figure 4.4**). Greater ChR2 expression may have allowed for larger increases in SWA power. Indeed, we found that optogenetic stimulation of anesthetized mice produced more robust, albeit non-specific, changes in LFP power when done in constitutively expressing mice. Given that our power was set to relatively low levels (3 mW/mm^2) at 40 ms pulses, the lack of expression may have contributed to lower peak and steady state photocurrents (Mattis et al., 2012). Secondly, higher frequencies may be more effective at mimicking the effects of SWA. This is in part supported by our anesthetized recordings, wherein we found that 1Hz stimulation - regardless of cohort - produced significant differences in LFP power within the SWA band while 0.5Hz failed to produce any significant change. These differences among experimental cohorts may have limited our ability to replicate the initial findings.

In addition to precluding significant changes in gene expression, we showed that SWA mimicry in sleep deprived *Ntsr1::ChR2* mice improved visual memory consolidation. Expression of SWA correlates with retention of declarative memories and performance in procedural tasks and its manipulation (via augmentation or disruption) reliably alters performance in various memory tasks (Puentes-Mestril et al., 2019). How SWA mediates memory consolidation is unclear. One line of research suggests that SWA coordinates interregional dialogue by coupling oscillating circuits. In other words, the temporal coupling of neocortical (SWA), hippocampal (theta, sharp wave ripples), and thalamic (spindle) oscillations allows for the transfer and subsequent storage of information- including visual information- between brain regions (Durkin et al., 2017; Maingret et al., 2016; Miyamoto et al., 2016; Rothschild et al., 2017; Wei et al., 2016). This dialogue may involve multiple forms of plasticity, including STDP, and it remains unclear how SWA's purported role in sleep dependent weakening would be expressed during coupling. Here we looked to establish whether SWA alone was enough to rescue behavior in a visual recognition task shown to be sleep dependent (**Figure 4.7**). Although we found that SWA mimicry improved visual recognition memory in sleep deprived *Ntsr1::ChR2* mice to wild-type control levels, we found no difference in performance between sleep and SD *Ntsr1::ChR2* mice. Subsequently, although SWA appeared to improve visual recognition, we cannot say that it rescued an SD-driven deficit.

There are important caveats in interpreting our results. Firstly, we found that a subset of mice underwent tonic-clonic seizures while undergoing optogenetic stimulation during SD (specifically at 3 Hz). These mice were not included in our final analysis. We did, however, include mice that had been subjected to different stimulation protocols (0.5-

3 Hz) at a previous time point, some of which underwent seizures. These mice were not statistically discernable from their counterparts (**Table 4.4**) and underwent no seizure on experiment day. Frequencies < 3Hz were found to be progressively less epileptogenic, with protocols at ≤ 1Hz producing no discernible seizures in naive mice. Consequently, we lowered our stimulation frequencies to either 0.5 Hz or 1 Hz. Because constitutively expressed Chr2 was found in all regions of the neocortex, we began virally transducing ChR2 in *Ntsr1-Cre* mice. Doing so allowed us to localize expression to V1, lessening the possibility of runaway excitation. No seizures were noted in these cohorts. Secondly, housekeeping instability may have inflated or reduced the changes in fold expression. Although we chose those housekeeping genes with relatively high stability rankings and low intergroup variance (according to our normfinder results), their expressions between conditions (**Table 4.1**) suggests some instability. Future studies may need to revisit housekeeping selection. Thirdly, our data is preliminary in nature. Although our initial results are promising, the lack of a YFP control group complicates interpretation, and our inability to replicate these results necessitates further investigation. Finally, some of the underlying assumptions in our experiments require further investigation. Whether rhythmic stimulation of layer 6 CT neurons drives true mimicry or simply alters cortical and thalamic firing is unclear. This is further complicated by the organizing properties of SWA. SWA, although largely characterized by delta, is known to coordinate the expression of other NREM rhythms including spindles and sharp wave ripples (SWRs) (Puentes-Mestril et al., 2019). Thus, only driving delta SWA may not truly mimic endogenous SWA. Moreover, it's unclear whether our visual recognition task reflects visual memory consolidation or is instead solely a measure of shifted tuning properties.

Changes in behavior may only reflect shifts in firing rate responses within V1 cells to specific oriented gratings (Aton et al., 2014). Consequently, they may reflect visual cortical firing properties and not necessarily memory consolidation. In addition, how or if this information is then relayed to the hippocampus is unknown. To address these ambiguities, future studies should limit mimicry of SWA to higher-order cortical regions critical to recognition memory, such as the perirhinal cortex (Murray et al., 1999). Studies should also supplement our oriented grating task with additional, better characterized, behaviors such as novel object recognition. Multi-unit recordings of targeted regions would also clarify the network effects of our optogenetic manipulation. A final issue, as underlined by our visual recognition task, is that constitutive expression of ChR2 in layer 6 CT neurons likely alters neuronal properties in unintended ways (Allen et al., 2015). Disaggregating the unintended effects of ChR2 expression from our results will be important in correctly interpreting our data. And future studies should use virally transduced mice when possible to lessen developmental effects of ChR2 expression. Nevertheless, we show that some components of sleep may be isolated and manipulated to better understand how sleep-dependent effects are realized.

Table 4.1. Housekeeping pairs used for all conditions. Change in gene expression presented as ratio[1] and fold change[2]

ChR2 Expression	Gene Set	Stimulation Frequency	SD(2-CT)/S(2-CT)[1]	Fold Change[2]
Constitutive	*Actg1+GusB+Pgk1*	No Stimulation	0.60	-1.65
		.5hz	N/A	N/A
		1Hz	0.98	-1.03
		3Hz	1.05	1.05
AAV Transduction	*Pgk1+Tbp*	No Stimulation	1.23	1.23
	GusB+Actg1	.5hz	0.97*	-1.03
	Pgk1+Tbp	1Hz	1.16	1.16

Table 4.2. Effect of SWA mimicry on IEG expression in constitutively expressing and AAV transduced *Ntsr1*-ChR2 mice

	Stimulated Hemisphere		Non-Stimulated Hemisphere		qPCR p-value (Stimulated vs. Not Stimulated)	
Gene Name	1Hz, ΔCT	3Hz, ΔCT	1Hz, ΔCT	3Hz, ΔCT	1Hz	3Hz
Arc	0.73 ± 0.31	0.65 ± 0.22	0.23 ± 0.09	0.20 ± 0.20	0.2139	0.2139
Homer1a	2.18 ± 0.14	2.21 ± 0.21	1.91 ± 0.11	2.03 ± 0.19	0.3977	0.4434

Table 4.3. Effect of SWA mimicry on IEG expression in stimulated and non-stimulated hemispheres

Gene Name	Sleep, ΔCT	SD, ΔCT	1Hz, ΔCT	3Hz, ΔCT	qPCR p-value (vs. Sleep)			qPCR p-value (vs. SD)	
					SD	1Hz	3Hz	1Hz	3Hz
Arc	1.65 ± 0.37	-0.26 ± 0.34	0.76 ± 0.31	0.65 ± 0.22	0.0094	0.1573	0.1573	0.1333	0.1573
Homer1a	2.22 ± 0.45	1.15 ± 0. 14	2.18 ± 0.14	2.21 ± 0.21	0.0398	0.9886	0.9886	0.0398	0.0398
Plk2	-0.32 ± 0.1	-0.17 ± 0.08	-0.27 ± 0.09	-0.4 ± 0.09	0.7659	0.8396	0.8396	0.8396	0.3805
Narp	3.20 ± 0.45	3.17 ± 0.39	3.25 ± 0.12	2.97 ± 0.47	0.9986	0.9986	0.9960	0.9986	0.9960
Gephyrin	2.47 ± 0.05	2.50 ± 0.04	2.66 ± 0.07	2.64 ± 0.08	0.7622	0.3155	0.3483	0.3483	0.3483
Gria1	0.44 ± 0.13	0.49 ± 0.08	-0.13 ± 0.23	0.31 ± 0.25	0.9079	0.2426	0.9079	0.2190	0.9079
Gria2	-2.36 ± 0.12	-2.23 ± 0.03	-2.10 ± 0.16	-2.03 ± 0.08	0.7376	0.5095	0.3804	0.7376	0.6545
Arc	1.15 ± 0.10	-0.63 ± 0.23	-0.49 ± 0.15	N/A	<0.0001	<0.0001	N/A	0.5568	N/A
Homer1a	1.73 ± 0.11	0.58 ± 0.16	0.59 ± 0.10	N/A	<0.0001	<0.0001	N/A	0.9515	N/A

Table 4.4. Effect of SWA mimicry on IEG expression in mice with and without a history of seizures

Gene Name	History of Seizure, ΔCT		No History of Seizure, ΔCT		qPCR p-value (Seizure vs. No Seizure)	
	Stimulated Hemisphere	Non-Stimulated Hemisphere	Stimulated Hemisphere	Non-Stimulated Hemisphere	Stimulated	Non-Stimulated
Arc	1.07 ± .03	0.27 ± 0.15	0.38 ± 0.48	0.19 ± 0.12	0.2985	0.8628
Homer1a	2.15 ± .29	2.08 ± 0.07	2.20 ± 0.13	1.73 ± 0.14	0.8360	0.3508

Table 4.5. Primer Designs

Gene Name	Forward Primer	Reverse Primer
Actg1	ACTCTTCCAGCCTTCCTTC	ATCTCCTTCTGCATCCTGTC
Pgk1	TCGTGATGAGGGTGGACTTC	ACAGCAGCCTTGATCCTTTG
Tbp	GCAGCCTCAGTACAGCAATC	GGTGCAGTGGTCAGAGTTTG
Gusb	TGGACCCAAGATACCGACAT	ATCCCATTCACCCACACAAC
Arc	CCAGATCCAGAACCACATGAA	GAGAGTGTACCCTCACTGTATTG
Homer1a	GCATTGCCATTTCCACATAGG	ATGAACTTCCATATTTATCCACCTTACTT
Plk2	AGATATAACGACACACACAATAAGGT	TTGCTAGGCTGCTGGGTTAT
Narp	TGCTGATAGAGTGGGGCAAT	CAGCTGTGCGACCTTGTC
Gephyrin	CAACAGGGAATGAGCTGCTA	CGATTGCTGTCCCGAATCTT
cFos	GAAGAGGAAGAGAAACGGAGAAT	CTTGGAGTGTATCTGTCAGCTC
Gria1	GGCCAGATTGTGAAGCTAGAA	ATGTCCATGAAGCCCAGGTT
Gria2	GATGCGACCTGACCTCAAAG	TGATAAGCCTCTGTCACTGTCA

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CHAPTER V

Conclusion and Future Directions

There were four objectives in this thesis. First, to characterize how sleep loss differentially alters the expression of genes involved in synaptic plasticity and circadian time keepers in a cell type- and region-specific manner. Second, to begin establishing a unifying theory for sleep's role in synaptic plasticity from commonalities shared by sleep oscillations. Third, to begin testing the relationship between SWA and sleep-dependent synaptic weakening through SWA mimicry. And lastly, to begin determining the functional relationship between SWA and visual memory consolidation. The summaries of our findings and future directions are discussed in the following sections.

5.1 Cell type- and region-specific variation in SD transcript regulation

Although sleep is known to play a critical role in learning and memory, the mechanism by which it does so remains largely unclear (Diekelmann and Born, 2010; Puentes-Mestril and Aton, 2017). Most hypotheses posit that sleep promotes synaptic plasticity mechanisms that stabilize acquired memories while readying the brain for further learning (Klinzing et al., 2019). To test these hypotheses, researchers have taken advantage of activity-dependent genes (IEG) and their relation to sleep, memory, and plasticity. Many IEGs are thought to mediate synaptic plasticity through increases in their expression following sleep loss

(Cirelli et al., 2004; Maret et al., 2007; Taishi et al., 2001; Terao et al., 2006; Tononi and Cirelli, 2006) and have been associated with memory consolidation (Fleischmann et al., 2003; Jaubert et al., 2007; Novkovic et al., 2015; Plath et al., 2006). In bridging sleep and synaptic plasticity, IEGs then offer a means of interrogating how sleep can facilitate memory through synaptic plasticity. However, researchers must consider the functional heterogeneity, cell-type specificity, and interplay of these IEGS. Most studies investigating sleep dependent gene expression have tended to focus on pyramidal cells or have circumscribed their analysis to single brain regions (Elliot et al., 2004). Recent data suggest that sleep dependent changes in gene expression do vary as a function of brain region and subregion (Calais et al., 2015; Delorme et al., 2019; Thompson et al., 2010), suggesting some heterogeneity in underlying mechanisms. In chapter II we looked to characterize how SD differentially impacts expression of commonly studied IEGs and circadian time keepers as a function of brain region and cell type.

In Chapter II, we used translating ribosome affinity purification (TRAP) to characterize changes in the abundance of ribosome-associated transcripts in Camk2a-expressing (Camk2a+) and parvalbumin-expressing (PV+) of the mouse hippocampus and neocortex after a brief period of sleep or sleep loss. We focused our analysis on sleep responsive IEGs implicated in various forms of synaptic plasticity. To better understand how IEG pathways may differ as a function of region and cell type, we looked at both effectors (*Arc, Homer1a, Narp, Bdnf*) and transcriptional regulators (*Cfos, FosB, Npas4*). In addition to these IEGs, we looked at core (*Clock, Per1, Per2, Cry1, Cry1, and Bmal*) and auxiliary (*Rev-Erba, Dbp, Ted, Nfil3, Dec1*) clock genes. We demonstrated that shorter (3-h SD) and more prolonged (6-h SD) sleep loss promoted distinct abundance

patterns in Camk2a+ and PV+ cells of the hippocampus and neocortex. Consistent with previous findings, 3-h SD significantly increased neocortical *Arc(Cirelli et al., 2004),* and *Homer1a* (Maret et al., 2008) abundance in Camk2a+ cells but only increased *Homer1a* abundance in the hippocampus. Transcript abundance for *Bdnf* and *Narp* followed a similar trend, with unchanged levels in hippocampal Camk2a+ neurons, and modest (but not significantly) increased levels in neocortical neurons. Prolonging SD to 6 h significantly increased ribosome-associated *Arc* and *Homer1a* abundance in Camk2a+ neurons of both regions, suggesting that hippocampal cells required additional SD before undergoing significant increases in IEG transcript abundance. Indeed, while both *Narp* and *BDNF* abundance significantly increased in the neocortex, only *BDNF* significantly changed in the hippocampal population. These results, circumscribed largely to excitatory cells of the hippocampus and neocortex, suggest some degree of regional variation, with hippocampal cells undergoing more modest increases in transcript abundance as a function of sleep loss.

When repeated these experiments in PV+ cells, we found that hippocampal PV+ interneurons were particularly refractory to SD-driven changes in transcript abundance while neocortical cells largely resembled their Camk2a+ counterparts. In the neocortex, short SD significantly increased *Arc* transcript abundance, while prolonged SD significantly increased *Arc*, *Homer1a*, *Narp*, and *BDNF* abundance. In contrast, we observed no significant changes in the hippocampus. Transcriptional regulators show cell type specific differences in expression (Hu et al., 2017; Hvratin et al., 2018; Lacar et al., 2016) and downstream targets (Lin et al., 2008). Regional differences in their expression would partially account for some of the differences observed. Subsequently, we repeated

our analysis with *Npas4*, *Cfos*, and *FosB*. We found that Camk2a+ neurons in the hippocampus underwent significant increases in *Npas4*, *Cfos*, and *FosB*. transcript abundance following prolonged SD. In contrast, only *Cfos* abundance increased in PV+ interneurons. These differences did not extend to neocortical populations, wherein all transcripts underwent significant increases in abundance. This suggests that hippocampal PV+ interneurons may have a higher induction threshold for IEG expression. This is supported in part by a recent study that found that the upregulation of CREB target genes (such as *Arc* and *Cfos*) in PV+ interneurons depended on the slower CaV1-CaMK-CaM pathway (Cohen et al., 2016). PV+ interneurons were generally found to be substantially less responsive to stimulation frequency and calcium influxes, resulting in slower overall kinetics. This is further supported by our short SD results, where we observed no significant change in transcript abundance within hippocampal PV+ interneurons but did find a significant increase in *Cfos* abundance in the Camk2a+ hippocampal population. In addition to slower transcriptional-activity coupling kinetics, lower PV+ interneuron activation may likewise account for differences in transcript abundance. Critically, we also found regional differences in transcript abundance within cell types, suggesting that region specific differences may be altering SD-driven abundance across cell types. Our data suggests that this may stem from lower overall hippocampal activity. We then shifted our analysis to core and auxiliary clock genes, we found similar (albeit more modest) variation in SD driven transcript abundance. 3-h SD significantly increased *Per2* transcript abundance in both Camk2a+ and PV+ neocortical populations but induced no change in the hippocampal populations. And, while prolonged SD (6-H) largely mirrored neocortical findings, with PER2 levels tending to remain

elevated across populations, it led to fairly divergent alterations in the hippocampus. Here we found that prolonged SD significantly altered abundance of ribosome-associated *Per2, Cry1*, and *Cry2* transcripts in Camk2a+ neurons (increasing *Per2* and *Cry1*, decreasing *Cry2*), while having no significant effect on transcript abundance in PV+ interneurons. Interestingly, our results suggest that SD-driven changes in clock (i.e. *Cry1*, *Cry2*, and *Per2)* transcript abundance can be selective, disrupting their usual pattern of expression across the 24-h cycle. Region and cell-type specific differences appeared greater among the auxiliary clock genes Here, we again found substantial heterogeneity in SD driven transcript abundance. Firstly, SD only impacted neocortical populations. Secondly, neocortical neurons responded in a cell-type specific manner. Short and prolonged SD promoted significant increases in neocortical *Nfil3* and *Dec1* transcript abundance only in PV+ cells and significantly decreased *Rev-Erb*$\square$ abundance only in Camk2a+ cells. Recent data suggests that some clock genes and IEGs share common signaling pathways involved sleep-dependent transcriptional regulation (Ingiosi et al., 2019; Sato et al., 2020). If so, regional differences in clock gene and IEG expression may stem from common or interacting pathways.

Built-in redundancy and crosstalk among transcription factors allows for the rapid upregulation of multiple IEGs through overlapping but distinct pathways. Effectors can themselves promote expression of distinct IEGs, further increasing the number of gene programs inducible by a single transcription factor. The potential for a high number of unique but overlapping IEG pathways is borne out by recent RNA single-cell sequencing studies that reveal surprising levels of cell type and subtype IEG variation (Hu et al., 2017; Hvratin et al., 2018; Lacar et al., 2016). This variation also may also serve as powerful

means of responding to network imbalances with tailored gene programs, further supported by recent work suggesting that IEG expressions act as transcriptional signatures of ensemble activity (Sun et al., 2020), reflecting underlying neuronal activity patterns with a high degree of specificity (Tyssowski et al., 2018; Tyssowski and Gray, 2019). Here, using translating ribosome affinity purification (TRAP), we've characterized changes in abundance of ribosome-associated transcripts in excitatory and inhibitory neurons in the mouse hippocampus and neocortex after a brief period of sleep or sleep loss. In doing so, we show that the effect of SD on IEG and clock transcript abundance is not uniform, but is instead more pronounced in excitatory neurons and cortical populations. This may reflect differences in underlying activity, resulting in divergent transcription patterns. Our data suggest that, insofar as abundance of all of these transcripts is regulated by neuronal activity (Yap and Greenberg, 2018), 1) neuronal activation in the hippocampus is reduced relative to neocortex during SD, and 2) PV+ interneuron activity and possibly transcription-activity coupling may vary less as a function of SD than Camk2a+ neuron activity. However, without electrophysiological recordings, we cannot definitively say that differences in overall activity are driving these differences. Future studies should combine FISH, qPCR, and single cell RNA sequencing with chronic in-vivo recordings of hippocampal and neocortical activity across sleep deprivation and *ad lib* sleep. Attempts should be made recording both putative excitatory and inhibitory cells. We could then correlate region-specific and cell-type specific differences in activity with IEG transcript abundance to find distinct transcriptional signatures driven by sleep loss.

We then interrogated whether total mRNA showed similar region- and cell type-specific differences in SD-driven expression, paralleling the changes in ribosome associated transcript abundance and supporting several recent findings (Delorme et al., 2019; Havekes and Aton, 2020). To do so we used fluorescence *in situ* hybridization (FISH) to quantify differences in effector (*Arc, Homer1a*) and transcriptional regulator (*Cfos*) IEG expression in C57Bl6/J mice following 6-h SD or *ad lib* sleep. This also allowed us to delineate sub regional differences in IEG expression across cortical layers 2/3-6 and hippocampal subregions Dentate Gyrus (DG), CA1,and CA3. To quantify expression among PV+ interneurons, we dichotomized our ROIs into *Pvalb* expressing (PV+ interneurons) and non-*Pvalb* expressing (all other cells) regions. Our findings largely supported the region- and cell-type specific changes in ribosome-associated transcript abundance. Whereas we found significant increases in *Arc*, *Homer1a*, and *Cfos* expression in non-PV+ cells throughout neocortical layers, neither *Arc* nor *Homer1a* levels were significantly altered by SD in any region of the dorsal hippocampus. Only *Cfos* expression in CA3 of the dorsal hippocampus was significantly altered by SD.In sharp contrast to the IEG expression found among non-PV+ neocortical cells, we observed low overall IEG expression in PV+ interneurons from all areas, suggesting that they were either less activated by SD or that their transcription-activity kinetics were substantially slower relative to their non-PV counterparts. Only *Cfos* expression in layer 2/3 of the neocortex was found to have increased following SD. Due to less total IEG expression, many PV+ interneurons expressed no detectable IEGs. These non-expressing PV+ interneurons may have hidden SD-driven increases in responsive PV+ interneurons. Consequently, we quantified expression within the subpopulation of PV+ interneurons

which had detectable levels of mRNA expression. We found that SD did not affect expression levels for *Arc* or *Cfos*, but did increase *Homer1a* dots/μm2 when measured across the entire neocortex. Critically, no significant changes in IEG expression were observed in PV+ interneurons in any region of dorsal hippocampus with SD. As a final measure of IEG expression in PV+ interneurons, we quantified the IEG+ proportion of PV+ interneurons in Sleep and SD mice as a function of cell count and ROI area. This would account for variable PV+ cell size. Here we found that the proportion of Arc+ and Cfos+ PV+ interneurons significantly increased for all neocortical layers quantified (with exception of Layer 5) and the whole neocortex. In support of our initial interpretation, no significant changes were found in the dorsal hippocampus.

Prior work has shown that SD can alter IEG expression in ways other than regulating *de novo* transcription. To better understand how sleep and sleep loss differentially impacts IEG expression in various regions and cell types, future studies should combine our experiments with an expanded target list of genes, including their pre-mRNA forms. Moreover, ribosome-associated transcripts are theoretically being prepared for translation. Their protein counterparts would thus offer a closer analog than total mRNA. Future studies should use immunohistochemistry to see if the above differences extend to protein expression.

5.2 Sleep brain rhythm regulation of plasticity-regulated transcripts and memory storage

The Synaptic Homeostasis Hypothesis (SHY) posits that sleep regulates synaptic strength via net synaptic weakening. According to SHY, during wakefulness synapses undergo strengthening that, if unregulated, can attenuate signal to noise ratios and disrupt memory consolidation (Tononi and Cirelli, 2014). During sleep, low frequency network oscillations (slow wave activity; SWA) are proposed to homeostatically weaken synapses to promote memory consolidation. Although partially supported by correlational data, no direct evidence of this function exists. And recent studies looking to characterize SWA's role in sleep dependent weakening have reported conflicting findings (Puentes-Mestril and Aton, 2017; Puentes-Mestril et al., 2019). In chapter IV we began to characterize the relationship between sleep-dependent weakening and SWA by manipulating SWA independently of behavioral state and characterizing subsequent changes in both IEG expression and sleep-associated memory consolidation.

To determine whether SWA is alone sufficient in producing sleep-dependent changes in gene expression, we rhythmically stimulated layer 6 corticothalamic (CT) neurons across 6-h SD in *Ntsr1*::ChR2 mice transgenic lines that constitutively express ChR2 in layer 6 CT neurons. Stimulation frequencies matched those previously shown to augment NREM oscillations in the delta (0.5-4 Hz) and spindle (7-15 Hz) frequency bands (Durkin et al., 2017). By rhythmically activating layer 6 CT neurons in these mice we are able to simulate SWA in a circuit known to coordinate these oscillations during sleep (Chauvette et al., 2010; Contreras et al., 1996; Timofeev and Steriade, 1996). Activating them may then allow us to better mimic natural SWA. We then compared gene expression

in SD stimulated, SD non-stimulated, and *ad lib* sleep mice. We focused our analysis on sleep responsive IEG effectors (*Arc, Homer1a, Narp*) previously implicated in homeostatic synaptic plasticity. In addition, we quantified the change in *Griar1* and *Gria2* expression - as additional proxies for synaptic plasticity - and *Plk2* and *Gephyrin* expression - as activity dependent genes likewise implicated in homeostatic synaptic plasticity. We found that rhythmically stimulating *Ntsr::Chr2* mice at 1 or 3 Hz blocked SD-driven increases in *Arc* and *Homer1a*. No other gene changed as a function of sleep or was significantly altered by optogenetic stimulation. These results suggest that we were able to mimic SWA and, in doing so, block the effects of sleep loss. That these effects were specific to *Arc* and *Homer1a* are important in three ways. Firstly, *Arc* and *Homer1a* are critical in the expression of homeostatic synaptic plasticity (Shepherd et al., 2006; Siddoway et al., 2014), with *Homer1a* having been extensively shown to be essential for downscaling (Hu et al., 2010), a phenomenon that closely resembles SHY. Secondly, knocking down either *Arc* or *Homer1a* significantly disrupts sleep homeostasis (Ahnaou et al., 2015; Suzuki et al., 2020). SWA is homeostatically regulated (Vyazovskiy et al., 2009), increasing in intensity across prolonged sleep loss and decreasing across a period of sleep. Shy posits that SWA mirrors sleep pressure because it is responsible for normalizing synaptic strengths across sleep (Tononi and Cirelli, 2014). Finally, knocking down *Homer1a* was recently shown to block sleep induced changes in synaptic composition indicative of synaptic weakening (Diering et al., 2017). If changes in *Homer1a* and *Arc* expression reflect homeostatic responses to wake associated potentiation, our data suggests that optogenetic mimicry of SWA was effective in block potentiation.

Unfortunately, our results come with many caveats. Firstly, we were unable to replicate these results when repeated at 0.5 Hz and 1 Hz in *Ntsr1-Cre* mice virally (AAV)-transduced to express ChR2. Electrophysiological recordings from anesthetized mice suggest that 0.5 Hz stimulation is not effective at mimicking SWA. And, while 1 Hz stimulation significantly increased LFP power in the SWA frequency band, we found that these increases were substantially larger in mice constitutively expressing ChR2 as opposed those virally transduced. These differences may explain why AAV-transduced mice were unaffected by optogenetic stimulation. Secondly, we found that a subset of *Ntsr1::ChR2* mice underwent seizures while stimulated at 3 Hz. Upon further investigation, it appeared that stimulation of layer 6 CT neurons at 3 Hz was particularly epileptogenic. Although we made efforts to ensure that our results did not include animals that underwent seizures on the day of the experiment, it still brings into question how our stimulation paradigm affects the underlying thalamocortical circuitry. We also found frequencies < 3 Hz to be progressively less epileptogenic, with protocols at ≤ 1Hz producing no discernible seizures in naive mice. For this reason, we capped our stimulation frequencies at 1 Hz and began exclusively using AAV-transduced mice. Using AAV transduction allowed us to localize expression of ChR2 to V1, theoretically lessening the possibility of runaway excitation. Finally, these results are still preliminary and need additional control and experimental groups to fully characterize how our manipulation is impacting cortical networks and whether it approximates naturally occurring SWA.

Mimicry of a naturally occurring oscillation presents many challenges and it remains unclear whether boosting cortical activity at SWA frequencies can effectively mimic the firing dynamics present during SWA. This is particularly important given recent

findings suggesting that altered firing dynamics during SWA can predispose neurons towards synaptic weakening (Bartram et al., 2017; Gonzalez-Rueda et al., 2018). Future studies should instead optogenetically disrupt SWA to determine whether it's required for sleep-dependent synaptic weakening. Our laboratory has shown that optogenetic inhibition of layer 6 CT neurons during NREM in *Ntsr1*::Arch3 disrupts neocortical SWA (**Figure 5.1**). If disruption of SWA in sleeping mice is found to promote higher *Arc* and *Homer1a* expression relative to non-stimulated mice, it would suggest that SWA is required to sleep dependent weakening and it would help clarify our initial findings.

In this study we also sought to determine if SWA is alone sufficient in rescuing behavior in a visual recognition task shown to be sleep dependent. Adapted from (Cooke et al., 2015), this task resembles a novel object recognition task but uses oriented gratings in lieu of physical objects. We first confirmed that this task was sleep-dependent, finding that SD effectively blocked recognition of the familiar grating, resulting in similar levels of interaction between the familiar and novel oriented gratings. We then rhythmically stimulated SD *Ntsr1*::*ChR2* mice at 3 Hz and compared their performance to non-stimulated SD and *ad lib* sleep groups. Our results were inconclusive but promising. Although we found that SWA mimicry improved performance of the sleep deprived *Ntsr1::ChR2* mice, increasing novel interaction to wild-type (WT) control levels, we found no significant difference in performance between non-stimulated *ad lib* sleep and SD *Ntsr1::ChR2* mice. Nevertheless, SWA appeared to improve visual recognition to levels parallel with WT control mice while sleeping groups, although not significant, tended to show greater preference for novel oriented gratings. There are two caveats that should be considered when interpreting these results. Firstly, although several studies suggest
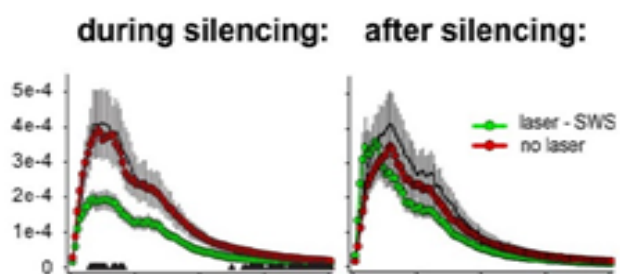
Figure 5.1: Optogenetic disruption of SWA. Left: SWS-specific inhibition of layer 6 corticothalamic neurons (**green**) results in significant attenuation of SWA (.5-4 Hz) during SWS, relative to control conditions shown in **red**. **Right:** Following cessation of optogenetic inhibition, SWA returns to baseline levels. p Indicates $p < 0.05$ in laser versus no laser condition.

that SWA facilitates memory consolidation, how it does remains unclear (Puentes-Mestril et al., 2019). One line of research suggests that SWA coordinates interregional dialogue by coupling oscillating circuits. Temporal coupling between neocortical (SWA), hippocampal (theta/Sharp Wave ripples), and thalamic (spindle) oscillations would allow for the transfer of information-including visual information- between brain regions and their subsequent storage (Durkin et al., 2017; Maingret et al., 2016; Miyamoto et al., 2016; Rothschild et al., 2017). Mimicking SWA may then be insufficient in rescuing visual memory in the absence of other oscillations. Secondly, it's unclear whether our visual recognition task reflects visual memory consolidation or is instead solely a measure of shifted tuning properties. Indeed, OSRP, the basis of our behavior, is measured as the change in firing rates responses to gratings of a specific orientation (Aton et al., 2014). How or if this information is then relayed to the hippocampus is unknown. Future studies should supplement behavior with chronic multi-unit recordings of targeted regions and use additional, better characterized, behaviors such as novel object recognition. This would clarify the effects of SWA mimicry as it relates to visual memory consolidation and network oscillations. Finally, future studies should target higher-order cortical regions, such as the perirhinal cortex, implicated in recognition memory (Murray et al., 1999). Targeted mimicry of SWA in these regions would address the ambiguities present in our novel orientation task.

The goals of this thesis have been to clarify the underlying mechanisms of sleep dependent synaptic weakening and to interrogate the assumption of uniform and global sleep dependent mechanisms. Additional studies should investigate specific examples of
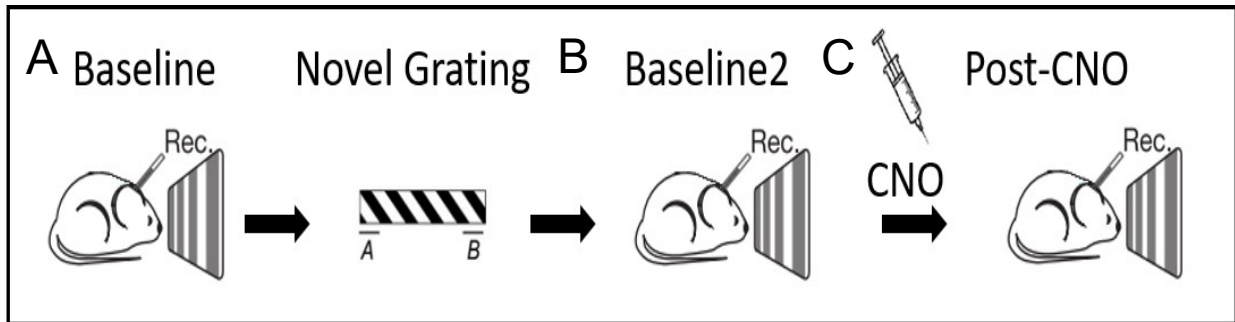
Figure 5.2 Future experimental design. Anesthetized mice expressing hM4Di within layer 6 corticothalamic neurons have LGN and V1 simultaneously recorded while undergoing visual stimulation to induce OSRP. **(A)** Baseline tuning curves are recorded to determine orientation preference, followed by 30 minute continuous presentation of a novel oriented grating. **(B)** Tuning curves are then re-measured to confirm successful OSRP induction. **(C)** Mice are given an intraperitoneal (i.p.) injection of CNO. Thirty minutes following CNO injection, tuning curves are measured again to assess the effect of disrupting V1-to-LGN communication.

sleep-dependent potentiation to better characterize the heterogeneity of sleep dependent mechanisms. A prime example is orientation specific response potentiation (OSRP), a form of sleep-dependent plasticity found within the primary visual cortex (V1) and lateral geniculate nucleus (LGN). As mentioned above, OSRP is elicited by the presentation of a novel visual stimulus (prolonged exposure to an oriented grating of specific orientation) and manifests as an increase in firing rate response to gratings of the same orientation during subsequent hours (Aton et al., 2014; Durkin and Aton, 2016). Critically, consolidation of OSRP in V1 appears to involve sleep-dependent synaptic strengthening, rather than weakening - a counterexample to the predictions of SHY. And its expression in V1 relies on NREM oscillations to relay information regarding orientation tuning from the LGN. Disruption of NREM oscillations, via optogenetic disruption of corticothalamic feedback from V1 to LGN, inhibits sleep-dependent consolidation of OSRP in V1. Using chemogenetics to alter its underlying circuit, future studies should characterize how SWA may mediate this form of sleep dependent plasticity and characterize concomitant changes in IEG expression to better understand how it may relate to other sleep-dependent phenomenon **(Figure 5.2)**.

5.3 Conclusion

In conclusion, the studies presented in this thesis advance our understanding of sleep by addressing two overarching questions, namely: 1) to what extent is sleep dependent weakening uniform and global and 2) what feature of sleep promotes synaptic weakening. Our TRAP study reveals significant heterogeneity in how sleep alters gene expression across cells types and regions. And begins to delineate subregional differences in neocortical and hippocampal SD-driven gene expression. Future studies

will need to characterize differences in neocortical and hippocampal activity during SD and correlate these differences to divergent gene expression patterns. Doing so will clarify the transcriptional signature of SD in these regions. As it stands, my thesis work challenges the predominant framework by which most of the sleep field operates: that sleep is a homogenous, global, and uniform process. Instead, sleep is heterogeneous, impacting regions and cell types in fairly divergent ways. Future research should reframe sleep-associated weakening as a heterogenous process with unknown levels of region and cell type specificity. Moreover, our optogenetic study begins delineating the role of SWA from other sleep components in sleep dependent synaptic plasticity and provides an experimental paradigm that can be built on. It is the first preliminary work to suggest that mimicry of SWA can replicate the effects of natural sleep in visual memory consolidation and IEG expression. In doing so, it offers a means of interrogating the mechanism underlying sleep-associated synaptic weakening. Future will need to refine these methods to better isolate SWA from sleep. Finally, in this thesis, we offer a thorough overview of sleep oscillations and their purported functions while presenting a unifying theory on oscillatory mediated synaptic plasticity. Overall, my thesis work significantly contributes to the field by challenging the SHY framework while offering a means of interrogating some it's proposed underlying mechanisms.

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