SLEEP AND CHRONOBIOLOGY IN PLASTICITY AND MEMORY

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SLEEP AND CHRONOBIOLOGY IN PLASTICITY AND MEMORY

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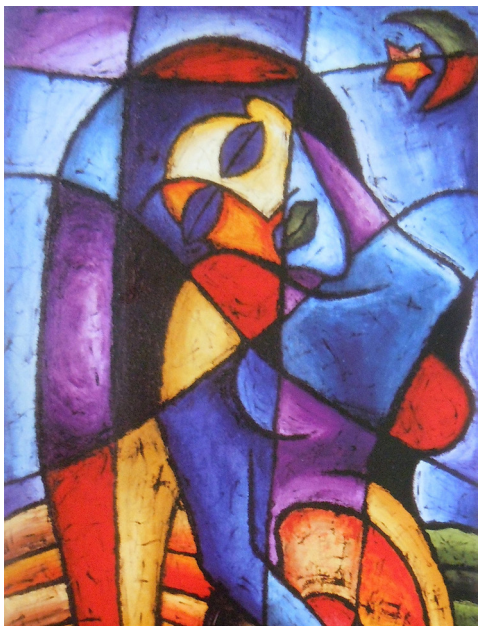

Chronobiological mechanisms regulating time-of-day mediated behaviors, such as sleep and circadian rhythms, are thought to interact with and/or share cellular and molecular signaling cascades that shape synaptic plasticity and neural excitability. These same factors are also known to underlie events that govern higher-order cognitive processing, including learning and memory formation, and often through phylogenetically conserved pathways. This suggests that factors which contribute to adaptive responses to changing environmental stimuli are likely derived from basic evolutionarily ancient processes, and underscores the importance of using both invertebrate and vertebrate models to study the interaction of chronobiology and cognitive processing. This issue highlights current views along with original research on sleep and circadian features of plasticity and memory in multiple species, models, and systems.

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EDITORIAL
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Waking up to the alarm: sleep, clocks, and making memory (s)tick

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Keywords: sleep, circadian rhythms, memory, plasticity, learning, synapse

In the immortal words of Woody Allen, "time is nature's way of keeping everything from happening at once." There is a time to work, a time to learn, and a time to rest. But in an increasingly 24-h society, the question of why *we must rest* comes up. Sleep and circadian rhythms influence brain plasticity-related processes, including neural excitability, synaptic efficacy, and cognitive abilities, such as learning and memory. How (and from an evolutionary perspective, why) sleep and the circadian clock have such influences over the brain is one of the great unsolved mysteries of biology. Clues regarding these interactions have been observed throughout the animal kingdom, and suggest basic mechanisms by which sleep and the circadian system that govern these processes are conserved phylogenetically. This Research Topic highlights current research and views on sleep and chronobiological features of plasticity and memory in multiple species, models, and systems. The authors present original research using invertebrate and vertebrate species, including moths, flies, rodents, and humans, giving the reader a broad understanding of available models and systems. Review articles discuss functional consequences of sleep and circadian disruption on cognitive processes, and survey current ideas within this burgeoning field of neuroscience. This Research Topic will hopefully stimulate more research inquiry and open the door for improving our understanding of relationships between sleep, chronobiology, and cognitive function.

Michel and Lyons (2014) underscore the importance of using invertebrate model systems for examining relationships between sleep, clocks, and memory. They show that vertebrate and invertebrate species, while separated by hundreds of millions of years of evolution, share common molecular, and cellular mechanisms that shape complex behavior and plasticity processes. Conservation of these basic mechanisms may have emerged out of ancient adaptive processes first directed by circadian processes to better equip species for survival, leading to testable hypotheses in multiple organisms (Gerstner, 2012). Available genetic and molecular tools, combined with strong phenotypes and cost-effectiveness, make invertebrate species powerful animal models for investigating mechanisms underlying complex behaviors, such as chronobiological aspects of memory formation.

Two original research articles harnessed the power of invertebrate model systems to reveal time-of-day effects on memory formation. Gage and Nighorn (2014) provide evidence for nitric oxide (NO) in the diurnal regulation of olfactory memory in the hawkmoth, *Manduca sexta*. Using the established proboscis extension reflex paradigm, a type of appetitive classical conditioning in *M. sexta*, the authors show NO-signaling has strong time-of-day effects on short- and intermediate-term memory formation. Fropf et al. (2014) use the olfactory avoidance conditioning paradigm in the fruit fly, *Drosophila melanogaster*, to characterize time-of-day effects on long-term memory formation. The authors show that these time-of-day dependent differences in memory performance are associated with changes in specific activation states of the protein dCreb2, a transcription factor implicated in sleep, circadian rhythms, and memory formation. These studies feature invertebrate models to characterize molecular signaling cascades which contribute to time-of-day dependent changes in memory formation, and lay groundwork for future studies to test whether similar pathways are conserved phylogenetically.

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Two original research articles employ studies in a mouse model system to assess how sleep and the clock regulate neurophysiology. Ognjanovski et al. (2014) present data on sleep- and wake-associated changes in CA1 hippocampal network activity during memory consolidation. While previous studies have described the necessity of sleep following single-trial contextual fear conditioning, the effects of conditioning and subsequent sleep on network activity have not been well understood. Here, Ognjanovski et al. (2014) show that consolidation of contextual fear memory is accompanied by heightened neuronal firing in the hippocampus. The authors observed that hippocampal network stability, as measured by functional connectivity analysis of neuronal spike trains, was greater after conditioning, specifically, during post-conditioning slow wave sleep, suggesting sleep may play a role in stabilizing patterns of neuronal communication following new learning. Gerstner et al. (2014) provide the first evidence that seizure threshold in mice is regulated by circadian clock mechanisms. Using a step-wise electroshock paradigm, the authors found that seizure thresholds peak in the early dark phase (the beginning of the active period), and that the core-clock gene BMAL1 is responsible for this effect, suggesting molecular clock mechanisms are able to regulate baseline neural excitability. Together, these data suggest that sleep and clock molecular factors are able to regulate neuronal network activity in mammalian brain, and provide novel models in rodents from which to explore mechanisms relating sleep and the clock in activity-dependent plasticity-related processes.

Two review articles elaborate on the use of rodent models for studying interactions between the clock, sleep, and brain function. Iyer et al. (2014) review comparisons between circadian plasticity mechanisms in the hippocampus and the master circadian pacemaker, the suprachiasmatic nucleus. The authors suggest circadian neuronal plasticity is gated by endogenous clock mechanisms, forming the basis for ∼24 h *iterative metaplasticity*, a term describing daily temporal confines to synaptic plasticity. Colavito et al. (2013) review rodent models in the study of sleep dependent memory processing. Here, the authors provide an extensive history on sleep deprivation methods to help facilitate interested researchers for developing customized laboratory protocols, and their application to preclinical testing. These articles highlight the use of rodent models to study interactions of sleep and circadian systems with brain plasticity and memory formation, and the potential for screening therapeutics in the treatment of cognitive disorders in humans.

This Research Topic also highlights recent findings in our understanding of sleep and the clock to human cognitive function. Gaggioni et al. (2014) summarize work that provides evidence for an interactive relationship of sleep homeostasis and circadian rhythmicity on cognitive brain activity in humans. Lemos et al. (2014) show naps are able to enhance memory in school-aged adolescents, evidence supporting sleep in facilitating memory. Finally, Bellesi et al. (2014) review work supporting the role of slow-wave sleep in cognitive performance, and provide an overview of methodological tools aimed at enhancing slow-waves in humans.

This Research Topic underscores the importance of using multiple model systems to broaden our understanding of the relationship between sleep, clocks, and memory. Basic mechanistic findings, taken from studies across species, will have important clinical relevance to our ever increasing 24-h society.

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REVIEW ARTICLE
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Unraveling the complexities of circadian and sleep interactions with memory formation through invertebrate research

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Across phylogeny, the endogenous biological clock has been recognized as providing adaptive advantages to organisms through coordination of physiological and behavioral processes. Recent research has emphasized the role of circadian modulation of memory in generating peaks and troughs in cognitive performance. The circadian clock along with homeostatic processes also regulates sleep, which itself impacts the formation and consolidation of memory. Thus, the circadian clock, sleep and memory form a triad with ongoing dynamic interactions. With technological advances and the development of a global 24/7 society, understanding the mechanisms underlying these connections becomes pivotal for development of therapeutic treatments for memory disorders and to address issues in cognitive performance arising from non-traditional work schedules. Invertebrate models, such as *Drosophila melanogaster* and the mollusks *Aplysia* and *Lymnaea*, have proven invaluable tools for identification of highly conserved molecular processes in memory. Recent research from invertebrate systems has outlined the influence of sleep and the circadian clock upon synaptic plasticity. In this review, we discuss the effects of the circadian clock and sleep on memory formation in invertebrates drawing attention to the potential of *in vivo* and *in vitro* approaches that harness the power of simple invertebrate systems to correlate individual cellular processes with complex behaviors. In conclusion, this review highlights how studies in invertebrates with relatively simple nervous systems can provide mechanistic insights into corresponding behaviors in higher organisms and can be used to outline possible therapeutic options to guide further targeted inquiry.

Keywords: circadian rhythms, learning and memory, sleep, *Aplysia*, invertebrates

SCOPE OF PROBLEM

In the past century, the nature of human society has been dramatically altered by technological innovations, communication advances, transportation improvements and urbanization. Non-traditional work schedules and round-the-clock manufacturing shifts have become increasingly common worldwide with the proportion of individuals working non-traditional work schedules rising. Recent research indicates approximately 3.7% of employed adult workers in the United States work a night shift with an additional 23.5% of individuals working non-traditional shifts including evening, rotating or split shifts (Luckhaupt, 2012; Alterman et al., 2013). Irregular work hours or jet lag desynchronize internal circadian oscillators that function to coordinate metabolic, physiological and behavioral processes in anticipation of daily environmental changes and orchestrate the timing of physiological and metabolic processes with behavioral activities.

Although the core circadian oscillator functions independently within individual cells, synchronization between oscillatory neurons and pacemakers is necessary to form functional circadian clocks for tissue and systems level rhythmicity (Albrecht, 2012). Neurons within the suprachiasmatic nucleus (SCN) are traditionally considered to comprise the master circadian clock in mammals. However, glial cells also have functional circadian oscillators and may modulate neuronal regulation of output rhythms (Prolo et al., 2005); for a review see Jackson (2011). Outside of the SCN, independent central circadian oscillators function within the olfactory bulb (Granados-Fuentes et al., 2004a,b, 2006) and rhythmic gene expression can be observed in multiple brain regions including the hippocampus (Holmes et al., 1995; Schaaf et al., 2000; Li et al., 2013). In mammals, as well as in lower vertebrates such as zebrafish (Whitmore et al., 2000) or invertebrates like *Drosophila* (Plautz et al., 1997), self-sustaining peripheral circadian oscillators can be found across multiple cell types and tissues including liver, heart, kidney, adrenal gland, pancreas and even fibroblasts (Balsalobre et al., 2000; Yoo et al., 2004), for reviews see Albrecht (2012) and Brown and Azzi (2013). These peripheral oscillators may be entrained at variable rates

or through mechanisms in addition to SCN signaling such as the time of food intake, body temperature, or metabolite and hormonal signaling (reviewed in Dibner et al., 2010; Albrecht, 2012; Mohawk et al., 2012), confounding the necessary resynchronization of oscillators between multiple tissues following jet-lag, irregular work hours or behaviors.

Technological advances have created the phenomenon of social jet lag for many age groups in which individuals significantly shift their wake/sleep cycles on weekends compared to the work week resulting in a bi-weekly activity dependent phase-shifting of the circadian clock (Wittmann et al., 2006; Roenneberg, 2013). Adolescents, with their circadian rhythms developmentally shifted toward late night chronotypes in particular are susceptible to social jet lag, which can result in negative health consequences and cognitive decrements (Collado Mateo et al., 2012; Touitou, 2013; Haraszti et al., 2014). The rising trends in the number of individuals affected by shift work, technological advances and social jet lag have resulted in an increasing proportion of the population that can be considered to have circadian dysfunction.

At the level of the individual, career and societal pressures often result in longer work days with extended temporal demands for high performance leaving less time for rest (Knutson et al., 2010; Roenneberg, 2013). Based on self-reported data collected through national surveys in 2005 and 2010, one-third of adult U.S. workers (approximately 40.6 million individuals) sleep 6 h or less at night (Cdc, C.F.D.C.a.P., 2005, 2007–2010). Although some differing conclusions exist between studies, in general it appears that individuals in the 21st century get significantly less rest compared to individuals 50–80 years ago, with rest time continuing to decrease over the last decade (Knutson et al., 2010; Bin et al., 2012; Luckhaupt, 2012; Roenneberg, 2013). Thus, the problems of sleep restriction, sleep disorders and circadian dysfunction appear pervasive in modern society.

Disturbances of the circadian clock through desynchronization or circadian dysfunction result in increased health problems for individuals with increased risk and incidence of metabolic diseases such as obesity and diabetes, cancer and heart disease as well as many other conditions (Barnard and Nolan, 2008; Preuss et al., 2008; Arble et al., 2010; Bass and Takahashi, 2010; Karatsoreos et al., 2011; Evans and Davidson, 2013; Orozco-Solis and Sassone-Corsi, 2014; Pluquet et al., 2014). Restricted sleep and sleep disorders also adversely impact individual health through increased risk of cardiovascular disease, immune system disorders, emotional and mood disorders, increased susceptibility to metabolic disorders, decreased cognitive performance and reduced quality of life and well-being (Breslau et al., 1996; Suka et al., 2003; Burgos et al., 2006; Neckelmann et al., 2007; Benca and Peterson, 2008; Goel et al., 2009; Vgontzas et al., 2009; Leproult and Van Cauter, 2010; Hsieh et al., 2011; Grandner et al., 2012). The increased incidence of automobile accidents, industrial accidents and occupational errors associated with sleep deprivation and sleep disorders raises the issue from the level of the individual to a societal crisis (Horne and Reyner, 1995; Pack et al., 1995; Lyznicki et al., 1998; Landrigan et al., 2004; Barger et al., 2005). Furthermore, decreased worker productivity leads to economic costs for businesses and industries with increased health problems exacerbating the loss in productivity by increasing health care costs to both employees and employers.

Independently of sleep deprivation or circadian dysfunction, the circadian clock itself modulates memory in humans and results in optimal times for memory and performance as well as trough phases in which significantly decreased performance in cognitive tasks occurs (Wright et al., 2002, 2006, 2012; Goel et al., 2013). This compounds the problems associated with shift work as circadian misalignment exists between the optimal times for cognitive performance and the phase in which some work is being performed. Moreover, when the effects of circadian phase such as work during a rest phase are compounded with extended time awake, the negative impacts on human cognitive performance are magnified (Dijk et al., 1992; Silva et al., 2010; Matthews et al., 2012b). Thus, the sleep restriction and fatigue faced by night shift workers, individuals in the transportation industry or health professionals, increase the problems associated with trying to optimize performance during sub-optimal circadian phases for cognitive performance. Additionally, the circadian clock can also modulate the impact of sleep deprivation on cognitive performance, further exacerbating the problem (Lo et al., 2012; Matthews et al., 2012a).

Despite the tremendous impact of sleep disorders and circadian dysfunction on individual health and society, the mechanisms and tri-partite interactions between sleep, the circadian clock and memory remain ill-defined at the synaptic or neuronal level. There is a continuing need for basic research investigating the underlying neural and molecular architecture involved in sleep, memory and circadian interactions in order to develop future treatments for disorders, improve cognitive performance, or design strategies to cope with the problems of shift work and social jet lag. Invertebrate research has proven to be fundamental in illuminating the basic principles and mechanisms underlying sleep, circadian rhythms and neuronal plasticity individually. In this review we will highlight recent advances that provide the framework and first steps to elucidate the interplay of these three research areas and discuss potential future directions.

INVERTEBRATES IN NEUROBIOLOGY RESEARCH

Given the considerable neuroanatomical differences between invertebrate models for neuroscience research (arthropods and mollusks) and higher organisms, differences in the levels of behavioral complexity, and the recent technological advances for targeted genetic studies in mammals, the reader may wonder whether research using invertebrate model systems will continue to advance progress in neuroscience research. Although vertebrate and invertebrate lineages diverged more than 900 million years ago (Peterson et al., 2004), the molecular and cellular mechanisms underlying neuronal behavior and synaptic plasticity are surprisingly well conserved across phylogeny. Understanding complex behaviors and the underlying cellular and molecular mechanism in higher organisms can be significantly facilitated through the study of these processes in comparatively less complex organisms.

Model systems such as *Drosophila melanogaster* and *Caenorhabditis elegans* have harnessed the power of neurogenetics

to dissect mechanisms underlying behavior. For example, research in *Drosophila* was key in identifying mechanisms underlying the core circadian oscillator (reviewed in Allada and Chung, 2010; Hardin, 2011; Ozkaya and Rosato, 2012) as well as the identification of signaling pathways underlying associative memory formation (McGuire et al., 2005; Davis, 2011) to name just two examples. *C. elegans* has furthermore emerged as a genetic model for studying memory and aging (reviewed in Murakami, 2007; Stein and Murphy, 2012; Chen et al., 2013; Sasakura and Mori, 2013). The tractability of these invertebrate model systems has been enhanced by powerful neurogenetic techniques that include forward genetic screens, reverse genetic techniques with genome-wide RNAi lines available, and optogenetics to assess individual neuronal changes using voltage or calcium sensors (reviewed in Sattelle and Buckingham, 2006). Recent research in alcohol neurobiology and drug addiction using *Drosophila* has demonstrated how insights gained from invertebrates can be leveraged into rapid advances in mammalian systems (Corl et al., 2009; Kaun et al., 2012; Kapfhamer et al., 2013). Likewise, in sleep studies, research using invertebrate models has advanced research in higher organisms as was elegantly shown by Paul Shaw and colleagues. In these studies, candidate genes for putative biomarkers of sleep loss were identified in human and rodent models and tested in *Drosophila*, subsequently facilitating further studies of additional biomarkers in mammals (Thimgan et al., 2013). The tools available for invertebrate research provide cost-effective, experimentally tractable systems for the rapid identification of novel pathways and cellular interactions associated with defined behaviors that can subsequently be investigated in more complex model systems.

The large size, determinate neuron position and the relatively small number of neurons in Molluscan species such as the marine mollusk *Aplysia californica* have proven invaluable for characterizing changes in cellular signaling pathways and synaptic plasticity associated with memory formation (Kandel, 2001; Bailey et al., 2008; Kandel et al., 2014). Likewise, studies in the freshwater pond snail *Lymnaea stagnalis* have led to important insights into the mechanisms of memory formation, particularly for the modulation of memory (Kemenes et al., 2006; Marra et al., 2013; Lukowiak et al., 2014). Moreover, these invertebrates have been pivotal in the initial recognition of non-synaptic forms of neuronal plasticity and their possible role in the neuronal representation of memory (Mozzachiodi and Byrne, 2010; Nikitin et al., 2013). Lastly, studies of neuronal injury and plasticity using molluscan models have expanded our understanding of chronic pain and other neurological disorders in humans (reviewed in Weragoda and Walters, 2007; Walters and Moroz, 2009; Crook et al., 2013).

Thus, research in invertebrates provides the ability to study system level interactions with broad impacts throughout the organism on physiological, behavioral and metabolic processes, such as the circadian clock, sleep, drug and alcohol use and neurological diseases. Although differing biological and evolutionary constraints may give rise to vital differences between invertebrate and vertebrate systems, an understanding of how evolution solved essential and complex conserved phenomena such as the interplay between memory and sleep in "simple" organisms will provide crucial insight into the molecular and cellular building blocks underlying these phenotypes in man.

SLEEP IN INVERTEBRATES

To unravel the interactions between the circadian clock, sleep and memory formation, it is necessary to have a model system in which all three processes interact. The repeated appearance of sleep across phylogenies suggests that sleep is an evolutionary necessity and its functions are conserved, if not its origins. In mammals and birds, sleep consists of two main stages, slow wave sleep and rapid eye movement sleep (REM), characterized by changes in neuronal activity as measured by electroencephalograms (reviewed in Rattenborg, 2006; Madan and Jha, 2012). Despite the similarities in the two stage sleep state of these groups, the emergence of REM and NREM sleep appears to be through convergent evolution (Rattenborg, 2007; Rattenborg et al., 2012). In aquatic mammals, reptiles, amphibians and other vertebrates, slow wave sleep has been consistently detected although REM sleep is not always present (Hobson et al., 1968; Mukhametov et al., 1977; González et al., 1999) reviewed in Williams et al. (1973) and Madan and Jha (2012). Monotremes, such as the echidna and platypus, demonstrate a single sleep state that shows characteristics of both slow wave and REM sleep (Siegel et al., 1996, 1998, 1999; Nicol et al., 2000). The uni-hemispheric presentation of sleep such as observed in dolphins (Mukhametov et al., 1977; Mukhametov, 1987; Sekiguchi and Kohshima, 2003) or the appearance of local sleep in sub-regions of the brain may explain the absence or minimization of REM sleep in aquatic mammals and other non-mammalian vertebrates (reviewed in Madan and Jha, 2012; Rattenborg et al., 2012). Local sleep with concurrent changes limited to specific neuronal groups complicates the investigation of the mechanisms underlying the interactions of sleep, the circadian clock and synaptic plasticity or memory formation in higher organisms. As a first step, it is necessary to study all components within the same circuit or neuronal network making the lower complexity of invertebrates attractive for research. However, this raises the question "do invertebrates sleep in an analogous manner to higher organisms?"

Invertebrate sleep is defined by means of behavioral characteristics including rhythmic activity and rest behaviors, characteristic rest body posture, preferred resting location, decreased responsiveness to sensory stimuli during rest (increased arousal thresholds) and rebound following rest deprivation (homeostasis) (Tobler, 1983; Hendricks et al., 2000; Zimmerman et al., 2008). As the regulation of sleep occurs dually through homeostatic processes and the circadian clock (Borbély and Achermann, 1999), interaction of the rest state with the circadian clock or expression of clock genes may also be used to further define sleep. Using some or all of these criteria, sleep or sleep-like states have been identified in dozens of invertebrates across phyla. **Table 1** highlights the activity phase and the type of sleep regulation observed for select invertebrate models that have also been used as models for studies of learning and memory or circadian research.

Recent advances using these relatively simple invertebrate systems have identified molecular and circuit mechanisms underlying sleep. For example, a sleep-like state has been reported for larval and adult stages of the nematode *C. elegans* in which

Table 1 | Examples of invertebrate species from the Phyla Mollusca and Arthropoda in which sleep has been studied.

	Organism	Activity Phase	Sleep characteristic	Reference
Phylum Mollusca	*Aplysia*	Diurnal	Homeostatic and Circadian Regulation	(Strumwasser, 1973; Vorster et al., in press)
	Lymnaea stagnalis (freshwater pond snail)	Greater Activity During Early Day	Sporadic Bouts, Regulation Unknown	(Wagatsuma et al., 2004; Aono et al., 2008; Stephenson and Lewis, 2011)
	Octopus	Nocturnal with pronounced dawn/dusk activity; may vary	Homeostatic and Circadian Regulation	(Brown et al., 2006; Meisel et al., 2006; Hochner, 2010)
	Sepia (Cuttlefish)	Diurnal, may vary	Homeostatic Regulation, Multiple Sleep-like States	(Duntley et al., 2002; Hanlon et al., 2007; Frank et al., 2012)
Phylum Arthropoda	*Drosophila melanogaster* (fruitfly)	Diurnal; Crepuscular	More sleep at night; Homeostatic and Circadian Regulation	(Hendricks et al., 2000; Shaw et al., 2000; van Alphen et al., 2013) reviewed in Bushey and Cirelli (2011) and Potdar and Sheeba (2013)
	Apis mellifera (honeybee)	Diurnal	Varies with worker caste and age; Multiple sleep stages; Homeostatic and Circadian Regulation	(Kaiser and Steiner-Kaiser, 1983; Kaiser, 1988; Sauer et al., 2003, 2004; Eban-Rothschild and Bloch, 2008, 2012; Klein et al., 2008)
	Leucophaea maderae; Blaberus giganteus (cockroach)	Nocturnal	Homeostatic and Circadian Regulation	(Tobler, 1983; Tobler and Neuner-Jehle, 1992; Decker et al., 2007; Garren et al., 2013)
	Procambrus clarkii (crayfish)	Diurnal	Slow wave brain activity during sleep, Homeostatic regulation	(Ramón et al., 2004; Mendoza-Angeles et al., 2010; Ramon et al., 2012)

quiescence is characterized by reduced sensory responsiveness, a characteristic rest body posture, and is timed through the expression of an ortholog of the canonical clock gene *period* (Jeon et al., 1999; You et al., 2008; Zimmerman et al., 2008; Iwanir et al., 2013; Nelson and Raizen, 2013). The simple wiring of the *C. elegans* nervous system allowed the identification of neuronal changes in sensory neurons and interneurons underlying the decreased arousal and quick reversibility of quiescence (Schwarz et al., 2011; Cho and Sternberg, 2014). At the molecular level, regulation of this sleep-like behavior has been associated with changes in the expression of highly conserved transcription factors (Driver et al., 2013; Turek et al., 2013) and neuropeptide signaling (Nelson and Raizen, 2013). The rapidity of research advances in the identification of the molecular and cellular mechanisms underlying sleep in *C. elegans* highlights the usefulness and potential for simple invertebrates in sleep research.

In insects, detailed studies of sleep have been performed in many species including cockroaches, bees, mosquitoes and *Drosophila* (Tobler, 1983; Tobler and Neuner-Jehle, 1992; Hendricks et al., 2000; Shaw et al., 2000; Eban-Rothschild and Bloch, 2008; Klein et al., 2008; Bushey et al., 2011). Over the past 15 years, our understanding of the functions of sleep and its functional necessity has rapidly progressed through research in *Drosophila* (reviewed in Cirelli and Tononi, 2008; Piscopo, 2009; Potdar and Sheeba, 2013) by providing evidence for the synaptic homeostasis hypothesis of sleep proposed by Tononi and Cirelli (2003). The synaptic homeostasis hypothesis is similar to synaptic scaling or homeostatic plasticity proposed for learning and memory (for a recent review see Schacher and Hu, 2014) and suggests that periods of activity with concomitant increases in synaptic strength are followed by synaptic downscaling during periods of sleep including decreases in synapse number (synaptic pruning) and weakening of synaptic connections (reviewed in Tononi and Cirelli, 2006, 2012). Structural evidence for synaptic pruning during sleep was recently provided in *Drosophila* (Bushey et al., 2011) along with the identification of sleep/wake changes in synaptic markers (Gilestro et al., 2009) consistent with the synaptic homeostasis hypothesis. *Drosophila* as a model has also been invaluable in advancing our understanding of the role of experience dependent plasticity in the need for sleep (Donlea et al., 2009) and the role of sleep in memory consolidation (Donlea et al., 2011). Furthermore, sleep in *Drosophila* also occurs in different stages with a deeper intensity sleep stage evident through recordings of brain activity (van Alphen et al., 2013). The rapid generation time of *Drosophila* combined with powerful neurogenetic approaches and forward genetic screens have allowed researchers to identify numerous genes and pathways regulating arousal and sleep (Koh et al., 2008; Chen et al., 2014; Park et al., 2014; Shi et al., 2014; Wu et al., 2014) as well as the timing of sleep onset (Liu et al., 2014). These factors have propelled *Drosophila* to the forefront of sleep research as a future model for screening and thereby understanding the genetic basis for sleep disorders in humans (Freeman et al., 2013). Despite these incredible and rapid advances in sleep research and the relatively small size of the *Drosophila* brain compared to mammalian brains, the complexity of the *Drosophila* nervous system combined with small neuronal size still poses some difficulties in decoding the tripartite interactions of sleep, the

circadian clock and memory formation within the same neuronal circuit.

In the morphologically diverse phylum Mollusca, conservation of sleep has been shown with sleep-like states characterized in Cephalopods and Gastropods. Cephalopods are often referred to as advanced invertebrates (Zullo and Hochner, 2011), as they have a highly developed centralized nervous systems and complex behaviors including higher order learning. *Octopus vulgaris* and the cuttlefish *Sepia* demonstrate sleep similar to vertebrate organisms with corresponding changes in behavior and differential brain activity (Duntley et al., 2002; Brown et al., 2006; Frank et al., 2012). However, increasing difficulties of working with cephalopods in laboratory settings and concerns for animal welfare (Fiorito et al., 2014) combined with the complexity of the cephalopod central nervous system make further studies of circadian and sleep interaction with memory or synaptic plasticity more difficult.

In comparison to cephalopods, gastropods are very different in morphological organization and development of the nervous system with a relatively simple central nervous systems and neurons clustered in distributed ganglia. Gastropods, including the terrestrial snail *Helix*, the pond snail *Lymnaea*, and the sea slugs *Hermissenda* and *Aplysia* have long been favorite models for neuroscience research and studies of synaptic plasticity. Anecdotal evidence and isolated literature references in early circadian and behavioral studies suggested resting or quiescent sleep states in *Aplysia* (Strumwasser, 1971, 1973; Preston and Lee, 1973), although systematic analysis of *Aplysia* sleep has only occurred recently. *Aplysia* sleep appears monophasic along circadian time with rest occurring only during the night. *Aplysia* further exhibit decreased sensory responsiveness during rest and sleep rebound following manual rest deprivation (Vorster et al., in press). Although the nervous system of the related gastropod *Lymnaea stagnalis* appears similar to *Aplysia*, surprisingly, rest-like states appear to be very different, with rest occurring infrequently and sporadically totaling less than 10% of the time (Stephenson and Lewis, 2011). Moreover, *Lymnaea* sleep does not appear to be regulated by the circadian clock, and rebound rest following deprivation has not been shown (Stephenson and Lewis, 2011; Stephenson, 2011). From these studies one could conclude either that *Lymnaea* require little sleep based upon their ecological niche or that sleep may be regulated via other mechanisms. Alternately, one may hypothesize that behavioral measures of sleep may not always be sufficient to characterize sleep and further investigations in simple invertebrates should be conducted to determine if sleep occurs at the level of the individual neuron. Although detailed characterization of the role of sleep in memory formation and synaptic plasticity has not yet been done in *Lymnaea* or *Aplysia*, the demonstration of sleep in these organisms highlights the potential for future research investigating the function and role of sleep in neuronal plasticity and memory formation using these classic neuroscience models.

THE CIRCADIAN CLOCK AND MEMORY

Energetically, neuronal and synaptic activities are expensive with the brain consuming about 20% of the total energy budget (reviewed in Harris et al., 2012). Behavioral evidence for the higher costs of neuronal activity and synaptic plasticity can be found in *Drosophila* (reviewed in Burns et al., 2011). Long-term memory formation is limited when starvation conditions exist (Plaçais and Preat, 2013) and memory formation decreases the resistance of flies to extreme stress (Mery and Kawecki, 2005). An evolutionary tradeoff exists in *Drosophila* between longevity and memory formation with longer-lived strains showing lower levels of memory (Burger et al., 2008; Lagasse et al., 2012). Likewise, selection of strains of flies with increased long-term memory results in decreases in longevity presumably due to the high metabolic costs associated with the improvements in memory and performance (Burger et al., 2008; Lagasse et al., 2012). One of the underlying tenets of the synaptic homeostasis hypothesis for sleep is that stronger synapses and strengthening of synapses during waking lead to higher energy consumption, that can be compensated for with synaptic downscaling during sleep which permits decreases in energy consumption allowing energy balance and restoration of cellular homeostasis (reviewed in Tononi and Cirelli, 2014). Thus while sleep functions as one mechanism for restoring energy balance, the energy demands associated with memory formation still exert a strong selective pressure for limiting memory formation as can be seen with the longevity studies in *Drosophila*.

One possible mechanism to restrict memory formation in concert with the animal's activity and physiology is through circadian modulation of memory. The far-reaching impact of the circadian clock on physiology can be observed through the adverse impacts associated with circadian dysfunction or desynchronization. Circadian dysfunction increases the risk of disease incidence for heart disease, metabolic diseases such as diabetes, multiple forms of cancer, mood disorders (reviewed in Barnard and Nolan, 2008; Preuss et al., 2008; Arble et al., 2010; Bass and Takahashi, 2010; Karatsoreos et al., 2011; Evans and Davidson, 2013; Orozco-Solis and Sassone-Corsi, 2014; Pluquet et al., 2014) as well as decrements in cognitive performance (Cho et al., 2000; Gibson et al., 2010; Loh et al., 2010). The broad scope of circadian modulation of metabolism and physiology suggests that there may be multiple levels through which the circadian clock could impact memory formation including sensory gating, enzymatic activity, intracellular signaling cascades, macromolecular synthesis or epigenetic regulation.

Interestingly, the mechanisms through which the circadian clock impacts memory appears to depend upon the type of learning, e.g., non-associative, classical or operant, and the type of memory formed, e.g., short, intermediate or long-term memory. Furthermore, the circadian clock may target memory through its induction, molecular consolidation, and/or recall. Focusing on invertebrate research, circadian modulation of memory has been observed in both insects and mollusks (see **Table 2** for examples). In cockroaches, short and long-term memories induced through classical olfactory conditioning are highly dependent upon time of training (Decker et al., 2007). Interestingly, during operant conditioning the circadian clock modulates recall, rather than memory formation, demonstrating how the circadian clock may regulate different steps in memory (Garren et al., 2013). In *Drosophila*, circadian modulation of olfactory conditioning has been shown directly for short-term

Table 2 | Examples of species frequently used for learning and memory studies in which sleep or circadian modulation of memory has also been examined.

	Organism	Learning Paradigm	Type of Modulation	Reference
Phylum Mollusca	*Aplysia*	Sensitization	Circadian modulation of intermediate and long-term memory formation, peak memory during the day	(Fernandez et al., 2003; Lyons et al., 2006a,b, 2008)
		Operant conditioning—feeding behavior	Circadian modulation of intermediate and long-term memory formation, peak memory during the day for diurnal *A. californica*. Peak long-term memory at night for nocturnal species	(Lyons et al., 2005, 2006b; Michel et al., 2013)
		Habituation, Classical Conditioning	Not Examined	Reviewed in Kandel (2001)
	Lymnaea stagnalis (pond snail)	Conditioned taste aversion	Diurnal modulation; peak learning in early morning	(Wagatsuma et al., 2004; Stephenson and Lewis, 2011)
	Octopus	Avoidance learning, Touch Discrimination, long-term potentiation	Not Examined	(Wells, 1965; Wells and Young, 1965; Hochner et al., 2003; Shomrat et al., 2008; Hochner, 2010)
	Sepia (Cuttlefish)	Spatial memory, associative memory	Not Examined	(Duntley et al., 2002; Alves et al., 2007; Frank et al., 2012; Cartron et al., 2013)
Phylum Arthropoda	*Drosophila melanogaster* (fruitfly)	Aversive phototactic suppression	Sleep deprivation affects short and long-term memory	(Seugnet et al., 2009, 2011)
		Courtship Conditioning	Sleep deprivation impacts long-term memory; increased sleep enhances long-term memory	(Ganguly-Fitzgerald et al., 2006; Donlea et al., 2011)
		Olfactory memory	Circadian and diurnal modulation of short and long-term memory	(Lyons and Roman, 2008; Fropf et al., 2014)
			Sleep deprivation affects short-term memory and long-term memory	(Li et al., 2009; Le Glou et al., 2012)
		Place preference	Decreased sleep impairs memory	(Bushey et al., 2007)
	Apis mellifera (honeybee)	Olfactory memory	Diurnal and circadian modulation of memory; Sleep deprivation affects extinction learning	(Hussaini et al., 2009; Lehmann et al., 2011)
		Spatial memory	Sleep deprivation affects memory consolidation	(Moore and Doherty, 2009; Moore et al., 2011; Beyaert et al., 2012)
	Leucophaea maderae (cockroach)	Olfactory memory	Circadian regulation of short and long-term memory; stage of modulation varies between classical and operant paradigms	(Tobler, 1983; Tobler and Neuner-Jehle, 1992; Decker et al., 2007; Garren et al., 2013)
	Manduca sexta (hawkmoth)	Olfactory memory	Diurnal regulation of short and intermediate-term memory	(Gage et al., 2013; Gage and Nighorn, 2014)
	Chasmagnathus (crab)	Habituation	Non-24 h intervals between training and testing impair long-term memory	(Pereyra et al., 1996)

and long-term memory (Lyons and Roman, 2008; Fropf et al., 2014) as well indirectly via circadian modulation of the vulnerability of anesthesia resistant memory to sleep deprivation (Le Glou et al., 2012). A recent study furthermore starts to unravel the molecular mechanisms by implicating circadian changes of a constitutive transcription factor well known for its role in long-term memory (Fropf et al., 2014). Core circadian clock genes also affect long-term memory formation as shown for courtship conditioning (Sakai et al., 2004). In honeybees, learning and memory are also modulated by the circadian timing of training (Lehmann et al., 2011). These studies in insects clearly demonstrate that circadian regulation of memory can

occur at multiple stages including the formation and recall of memory.

In addition to the circadian clock itself modulating memory or recall, core circadian genes also function in synaptic plasticity outside of their roles in the circadian oscillator. Independent of its canonical role in the *Drosophila* central pacemaker, the blue-light sensor CRYPTOCHROME (CRY) has the ability to mediate neuronal firing rates in response to light through potassium channel conductance (Fogle et al., 2011). Furthermore, the core clock protein PERIOD (PER) appears necessary for robust long-term memory as *per* mutant flies exhibit deficits in conditioned courtship memory (Sakai et al., 2004). Similar complexity in circadian regulation of memory at multiple levels has also been observed in vertebrates (reviewed in Gerstner and Yin, 2010; Jilg et al., 2010; Bechstein et al., 2014).

Circadian modulation of memory dependent upon the time of training has also been shown for intermediate and long-term memory in mollusks where the most extensive studies have been conducted in *Aplysia* (reviewed in Lyons, 2011). Using a non-associative form of learning, sensitization of the tail-siphon withdrawal reflex, it was found that both protein synthesis dependent intermediate-term memory and long-term memory were regulated by the circadian clock dependent upon time of training (Fernandez et al., 2003; Lyons et al., 2008). Although long-term memory in this case appears to be regulated only during its induction and formation, multiple processes are regulated by the circadian clock following training including neurotransmitter release, the induction of MAPK signaling and immediate early gene expression (Lyons et al., 2006a). The circadian clock also modulates intermediate and long-term memory for an associative operant learning paradigm (Lyons et al., 2005; Michel et al., 2013). In contrast to the circadian regulation of short-term memory observed in insects, *Aplysia* short-term memory for either associative or non-associative paradigms does not appear to be regulated by the circadian clock (Fernandez et al., 2003; Lyons et al., 2005), perhaps due to the specific ethological relevance of the learning paradigms used in these studies, a defensive reflex and feeding behavior. In *Lymnaea*, time of day also affects the acquisition of memory for conditioned taste aversion (Wagatsuma et al., 2004). The appearance of strong circadian modulation of memory across species and learning paradigms demonstrates that this modulation provides a clear evolutionary advantage. Recently, studies in rodents have shown that the reverse modulation also occurs, i.e., that memory formation can impact the circadian timekeeping of the organism. Using a hippocampal dependent learning paradigm and sustained activity tasks, researchers demonstrated that training inducing memory formation conducted during the animal's inactive period resulted in a phase shift in locomotor activity (Gritton et al., 2012). Although the potential bidirectional interaction of memory formation and synaptic activity on the phase of the circadian clock have not been investigated using invertebrate models, understanding the impact of memory formation anti-phase to activity cycles will be important for managing the issues of memory and performance associated with shift work. Thus, it will be of considerable interest to identify the molecular mechanisms of this interaction as well as the parameters and circumstances under which these bi-directional influences occur. Invertebrate models provide excellent systems for investigating the forward modulation of memory by the circadian clock and identifying the types and mechanisms through which memory or cognitive activity impact the circadian oscillator or other circadian rhythms.

FUTURE DIRECTIONS

Identifying the individual neurons and neural networks causal to complex behaviors and studying the interactions between multiple processes such as memory, sleep and the clock is one of the greatest challenges in modern neuroscience. Using invertebrate model systems, it becomes feasible to investigate the interactions of sleep, the circadian clock and synaptic plasticity at the level of individual neurons or neuronal circuits permitting the possibility of addressing the following topics: (1) determine how individual neurons or neuronal circuits involved in memory formation are stabilized through time, reactivated, undergo periods of malleability and degradation/pruning; (2) determine how the effects of sleep as a network property or at the level of individual neurons affects neuronal plasticity; (3) determine how the core circadian clock regulates sleep in individual neurons and circuits; (4) determine how sleep and circadian time modulate neuronal and synaptic activity for specific tasks or memories; (5) determine how effects analogous to modern life such as non-traditional sleep/wake cycles or shifted circadian rhythms affect this interplay transiently and chronically; and ultimately; (6) identify mechanisms through which these interactions can be modified by drugs or optimized behavioral patterns.

In support of this, recent studies in invertebrates have identified specific neurons that regulate sleep (Donlea et al., 2014) as well as a circadian transcript that modulates sleep onset in *Drosophila* (Liu et al., 2014). Sleep has also been shown to regulate net synaptic homeostasis, thereby "resetting" the nervous system to allow future plasticity (Tononi and Cirelli, 2014). From this and the studies mentioned in the previous sections it follows that the molecular and cellular substrate for the interplay between memory, sleep and the clock is slowly crystallizing from invertebrate studies, and this will significantly inform follow up research in mammalian and simian species.

One of the greatest benefits of the molluscan model system is the spectrum of techniques permitting an integration of top-down (*in-vivo*) and bottom-up (*in vitro*) approaches in the experimental design and the exquisite detail possible with investigations at the level of individual neurons or neuronal circuits. In gastropod mollusks, the precisely wired nervous system is comprised of large neurons with determinate location and in many cases functions of individual neurons identified (see for example Benjamin et al., 2000; Moroz, 2000; Kandel, 2001; Kandel et al., 2014). In models such as *Lymnaea* and *Aplysia*, the advantages of multilevel approaches combining *in vivo* behavioral studies with *in vitro* cellular studies to correlate complex behaviors with individual neuronal behavior have been well-documented.

For example in *Aplysia*, long-term facilitation provides a cellular correlate for behavioral sensitization that can be investigated in reduced preparations and cultured neurons (see for example Kandel, 2001; Hawkins et al., 2006). In a semi-intact reduced preparation in which the siphon and tail are exposed with the

nerves connecting the ganglia left intact, intermediate and long-term sensitization can be studied for either the tail-siphon withdrawal reflex or for a tail-tail withdrawal reflex (Philips et al., 2006, 2011). The existence of extra-ocular photoreceptors and extra-ocular circadian oscillators involved in circadian modulation of memory in *Aplysia* (Lyons et al., 2006b) widens the potential for studying system level interactions between the circadian clock and memory formation using semi-intact reduced or whole ganglia preparations. Furthermore, sleep has been characterized in *Aplysia* that demonstrates changes in sensory arousal accompanying sleep and homeostatic rebound rest following sleep deprivation (Vorster et al., in press). Individual neuronal changes correlated with behavioral changes can be identified using well-developed whole mount immunohistochemical and *in situ* techniques as shown in *Aplysia* (Sweedler et al., 2002; Jezzini et al., 2005). The neurons known to underlie behavioral effects seen *in vivo* as well as in the reduced preparation can further be co-cultured in a dish (Cai et al., 2012), or a compartmentalized dish to separately investigate synaptic vs. somatic changes within the neurons (Ye et al., 2012). Moreover, the large size of *Aplysia* neurons and determinate neuronal position also allows for genomic and metabolomic analysis at the scale of the individual neuron (Moroz and Kohn, 2013; Nemes et al., 2013), or even the synaptic transcriptome (Puthanveettil et al., 2013).

Similar anatomical advantages for studies of individual neurons or neuronal circuits exist in *Lymnaea* in which multi-electrode arrays have been used in order to record from up to 60 electrodes simultaneously (Harris et al., 2010). Additionally, the large neuronal size permits investigation of plasticity at subcellular levels using behavioral, electrophysiological and optical recording techniques. For example, recent research in *Lymnaea* demonstrated that learning-induced non-synaptic plasticity contributed to specific, compartmentalized presynaptic changes through calcium signaling (Nikitin et al., 2013). The advantages of large neuronal size and the potential for individual neuronal manipulations through pharmacological or electrophysiological methods combined with the diversity of recording techniques ensures that molluscan neuronal circuits will remain at the forefront of our progress in studies of synaptic plasticity and neuronal behavior.

Given the availability of abundant data underlying neuronal network function in molluscs, mathematical modeling can be leveraged to drive further experimental inquiry (Vavoulis et al., 2007; Liu et al., 2013). Importantly, the incorporation of predictive neuronal networks to direct experimental investigations facilitates the identification of key signaling molecules in behavior and methods to ameliorate conditions caused by mutations in these pathways. In a set of elegant studies, the Byrne lab has modeled the molecular underpinnings of *Aplysia* memory processes to develop a training paradigm predicted to lead to a stronger induction of the molecular cascades necessary for memory. Testing these predictions behaviorally, they found that the modeled training protocols resulted in stronger and longer lasting memory (Zhang et al., 2011). In subsequent studies, the power of computer simulations was leveraged to predict an ameliorated training paradigm to overcome the knockdown of a protein commonly mutated in Rubinstein-Taybi syndrome, strongly suggesting that modeling using the molecular and circuit level insight gained from invertebrates can be used to predict optimal learning strategies potentially in lieu of or in conjunction with drug therapy (Liu et al., 2013).

The multi-level combinatorial approaches outlined above set the stage to yield unprecedented insight into the mechanisms through which sleep or the circadian clock affects neuronal plasticity. The high degree of phylogenetic conservation as previously discussed permits the rapid translation of research in invertebrate systems to mammalian models (see also Abrams, 2012). It stands to reason that even with evolutionary differences resulting in non-conserved transcripts between invertebrates and mammals, the role that these molecules play will have an analogous function in mammals since the circadian clock, sleep and memory are evolutionarily well-conserved processes. Taken together, research in relatively simple mollusks such as *Aplysia* presents the scientific community with a powerful toolset to identify the cellular and molecular pathways from the whole animal to mathematical models, laying the foundation to identify the basic principles and mechanisms of memory modulation. Unraveling the intricate relationships between sleep, the circadian clock and learning and memory is essential to identify key molecules or interacting nodes for the development of future therapies to improve cognitive performance and decrements associated with shift work, sleep disorders, aging or neurological disorders.

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The role of nitric oxide in memory is modulated by diurnal time

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Nitric oxide (NO) is thought to play an important neuromodulatory role in the olfactory system. This modulation has been suggested to be particularly important for olfactory learning and memory in the antennal lobe (the primary olfactory network in invertebrates). We are using the hawkmoth, *Manduca sexta*, to further investigate the role of NO in olfactory memory. Recent findings suggest that NO affects short-term memory traces and that NO concentration fluctuates with the light cycle. This gives rise to the hypothesis that NO may be involved in the connection between memory and circadian rhythms. In this study, we explore the role of diurnal time and NO in memory by altering the time of day when associative-olfactory conditioning is performed. We find a strong effect of NO on short-term memory, and two surprising effects of diurnal time. We find that (1) at certain time points, NO affects longer traces of memory in addition to short-term memory; and (2) when conditioning is performed close to the light cycle switches—both from light to dark and dark to light—NO does not significantly affect memory at all. These findings suggest an intriguing functional role for NO in olfactory conditioning that is modulated as a function of diurnal time.

Keywords: moth, olfaction, antennal lobe, classical conditioning, proboscis extension

INTRODUCTION

Nitric oxide (NO) is a free radical gas that can serve as both unconventional neurotransmitter and neuromodulator. Nitric oxide synthase (NOS) is highly expressed in the primary olfactory center in both vertebrates (olfactory bulb) and invertebrates (antennal lobe) (Bredt et al., 1991; Elphick et al., 1995; Muller and Hildebrandt, 1995; Hopkins et al., 1996; Kendrick et al., 1997; Nighorn et al., 1998; Fujie et al., 2002; Collmann et al., 2004). Given this widespread prominence, NO is thought to play a functional role in olfactory processing and behavior, yet the significance of this role is only beginning to be understood. We have previously shown that NO is necessary for short-term olfactory memory in the AL in the moth, *Manduca sexta* (Gage et al., 2013). These moths are nocturnal and heavily depend on their olfactory systems to find mates, feed, and find sites to lay eggs. During the nocturnal active period, NO levels are significantly higher in the antennal and optic lobes, suggesting that NO signaling is heightened at night and may play a phase-dependent role.

Circadian influence on memory and behavior is highly conserved (Gerstner et al., 2009). The repetitive nature of the light cycle that coincides with the availability of vital resources has led to a "timed" physiological environment (Gerstner, 2012). In this way, organisms experience physiological changes at the cellular and molecular level that are both circadian and seasonal, and ultimately lead to timed variations in behavior. These behavioral responses are often coordinated with regular and predictable stimuli present in the environment. For example, bees and moths forage at the time of day when pollen and sucrose are at peak levels (Baker, 1961; Guerenstein et al., 2004). Memory, which is intricately intertwined in behavior, has also evolved in a circadian context. Learning and memory are metabolically expensive, and it is widely believed that these mechanisms are conserved and function optimally when predictable resources are available (Dukas, 2008; Lyons, 2011; Gerstner, 2012). In essence, there is a "plasticity in plasticity".

Though behavior and memory are controlled by the circadian clock, the nervous system can also modulate these effects (Gerstner, 2012). This variation is believed to exist to help animals adapt to a changing environment, such as the change in daylight hours throughout the year. This ability to adapt is suggested to be regulated by neuromodulators (Gerstner, 2012). Neuromodulators adjust sensory circuitry to account for changing conditions and are thought to optimize energy use in finding resources. NO could be an important neuromodulator in this process. NO is demonstrated to affect memory in many species and paradigms (Yamada et al., 1995; Muller, 1996; Kendrick et al., 1997; Prendergast et al., 1997a,b; Samama and Boehm, 1999; Yeh and Powers, 2005; Yabumoto et al., 2008; Kelley et al., 2010; Mutlu et al., 2011), and some reports also find direct effects of NO in the superchiasmatic nucleus (Ignarro, 2000) and in peripheral pacemakers (Bullmann and Stevenson, 2010).

The olfactory system provides an excellent opportunity to investigate the role of NO in memory. The primary olfactory center is organized similarly across phylogeny (Hildebrand and Shepherd, 1997), and NO is highly expressed in every primary

olfactory center in which it has been examined (Bredt et al., 1991; Elphick et al., 1995; Muller and Hildebrandt, 1995; Hopkins et al., 1996; Kendrick et al., 1997; Nighorn et al., 1998; Fujie et al., 2002; Collmann et al., 2004). Olfactory learning and memory, especially in insects, is well-studied, and much is known about the behavior and molecular components (for reviews see Dukas, 2008; Davis, 2011; Giurfa and Sandoz, 2012). Olfactory memory also appears to be regulated by the circadian clock. Several reports reveal a circadian-dependent change in memory using olfactory conditioning. These effects have been demonstrated in the cockroach, *Leucophaea maderae* (Decker et al., 2007); in the soil dwelling nematode, *Caenorhabditis elegans* (Olmedo et al., 2012); and in the fruit fly, *D. melanogaster* (Lyons and Roman, 2008). It appears that the circadian clock regulates memory rather than olfactory responsiveness (Lyons and Roman, 2008; Lyons, 2011). Studies in rodents show that olfactory bulb neurons express functional and entrainable circadian rhythms that operate independently of the superchiasmatic nucleus (Granados-Fuentes et al., 2004). These rhythms in olfactory activity in both vertebrates and invertebrates appear to depend on *BMAL1* and *period* genes (Krishnan et al., 1999; Tanoue et al., 2004; Lyons and Roman, 2008; Granados-Fuentes et al., 2011; Hamada et al., 2011).

In this study, we utilize the olfactory system of *M. sexta* to study the role of NO in memory in relation to diurnal time. *M. sexta* demonstrate robust learning and memory in classical conditioning paradigms using the proboscis extension reflex (PER; Daly and Smith, 2000; Dacks et al., 2012; Gage et al., 2013). The olfactory behavior and ecology in the hawkmoth is well described and can be useful when interpreting olfactory memory with light/activity phase effects (Baker, 1961; Grant, 1983; Riffell et al., 2008). Although *M. sexta* is not a traditional model used in circadian rhythm biology, *period* expression is found in the photoreceptors in the compound eye, neurons in the optic lobe, and glial cells in the AL (Wise et al., 2002). We know that NOS is localized in the olfactory receptor neurons and sGC is expressed in all projection neurons, some local interneurons, and the serotonin immunoreactive neuron (Collmann et al., 2004). NO also exerts substantial effects at the physiological level in *M. sexta* that include: (1) a spatially focused increase in NO during odor stimulation (Collmann et al., 2004); (2) persistent basal levels in olfactory neurons that affect resting membrane conductance (Wilson et al., 2007); and (3) whole-cell current modulation (Higgins et al., 2012).

We ask two main questions in this study: (1) is there an optimal time of day for learning and memory in *M. sexta*; and (2) does the role of NO in memory change depending on the time of conditioning? To do so, we pair a microinjection surgery to manipulate NO levels in the AL with an appetitive, odor-associative conditioning paradigm. Conditioning is performed at different times around the clock that include 12 h of day, followed by 12 h of night. The PER is used to measure memory of the conditioned odor (CS). We tested olfactory memory at four time points after conditioning to account for both short-term, intermediate-term, and long-term memory traces. We present findings that suggest a role for NO in short-term and intermediate-term memory in *M. sexta* that is modulated by diurnal time.

MATERIALS AND METHODS

ANIMALS

M. sexta (Lepidoptera: Sphingidae) were reared in the Department of Neuroscience at the University of Arizona. Animals were raised on an artificial diet and maintained under a long-day photoperiod regimen (17 h light:7 h dark) at 25°C at 50–60% relative humidity until early in pupal development. Most of pupal development occurs in constant darkness. Females at pupae stage 16 were transferred into a biological incubator (Model I-36 VL; Percival Scientific, Perry, IA, USA) and placed under a 12 h light:12 h dark cycle and kept at 25°C at 50–60% relative humidity. Five-day-old females were unfed after eclosion and used for all experiments. For all experiments, each animal was used only once.

PHARMACOLOGY AND MICROINJECTION SURGERY

NOS inhibitor, *N*-nitro-L-arginine methyl ester (L-NAME), was dissolved into physiological saline (150 mM NaCl, 3 mM KCl, 10 mM TES, pH 6.9) and used at a 15 mM concentration. L-NAME was chosen because it is the best characterized NOS inhibitor in this system. In *M. sexta*, this concentration was found to be the minimal effective dose in extracellular recording (Wilson et al., 2007) and also found to affect odor learning and memory (Gage et al., 2013).

Drug delivery into the ALs was accomplished via a microinjection surgery (Lei et al., 2009; Gage et al., 2013). Animals were restrained in a plastic tube, and an hourglass window was cut into the head capsule. The ALs were visualized by gently moving connective tissue with fine forceps. Quartz pipettes (o.d. 1.0 mm, i.d. 70 mm; Sutter Instruments, San Diego, CA, USA) were pulled with a Model P-2000 puller (Sutter Instruments) and clipped to allow solution passage. The pipettes were filled with either L-NAME or saline and manually injected into each AL (for visual see Gage et al., 2013) using a General Valve Picospritzer II (East Hanover, NJ, USA). The cut window was resealed with myristic acid (Sigma-Aldrich). The identity of the drug *versus* saline control was blind to both the experimenter performing the surgery and the experimenter observing behavior in all experiments.

In these experiments, NO levels were manipulated by inhibiting NOS rather than providing an exogenous source of NO. This is due to the unique glomerular anatomy of the olfactory system. In *M. sexta*, the glial cells surrounding each glomerulus provide a strong barrier to diffusion of NO making the bath application of a NO donor potentially problematic (Collmann et al., 2004; Higgins et al., 2012).

OLFACTORY STIMULATION AND APPETITIVE CONDITIONING

Hibiscus oil blend (diluted 1:1000 in mineral oil; Select Oils, Tulsa, OK, USA) was the odor used for appetitive conditioning. Hibiscus is not a reported host plant of hawkmoths and serves as a novel odor to gauge odor-associative learning and memory. Hibiscus was delivered by a solenoid-controlled air stream into an odor-containing glass syringe. Each syringe contained 10 μL of the odor on a piece of filter paper.

Appetitive conditioning was performed utilizing the PER. This is a feeding reflex that was originally discovered in honeybees

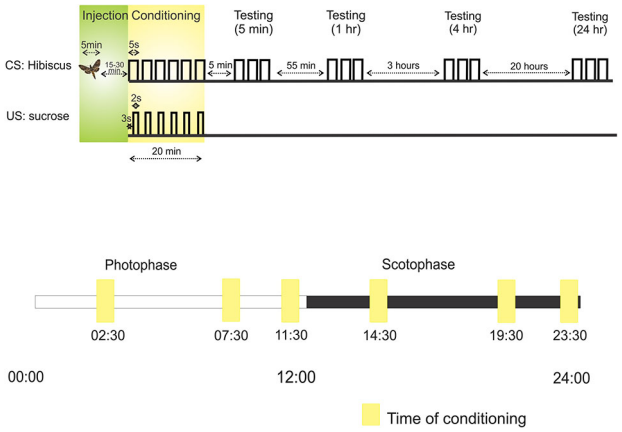

FIGURE 1 | Protocols outlining the sequence of injection, conditioning and testing. Above: The first line, *CS: Hibiscus*, denotes the presentation of the conditioned stimulus (CS), hibiscus odor blend, through an air puff. Each raised step on this line refers to the presentation of hibiscus. For example, during conditioning, hibiscus was presented a total of six times to the antenna. The second line, US: sucrose, represents when sucrose, the unconditioned stimulus (US), was applied to the tip of proboscis relative to the CS odor puff. For example, sucrose was applied 3 s into the odor puff. Below: the protocol for testing learning and memory at different times over a 24 h period. Each square represents the time of conditioning in six separate experiments. Scotophase, represented by the black bar, denotes nighttime and photophase represented by the white bar, denotes daytime.

(Takeda, 1961) that has also been used in *M. sexta* (Daly and Smith, 2000). Moths trained to associate an odor with a sucrose reward will extend their proboscis to the rewarded odor. This measure can be used in a number of paradigms and is especially useful to gauge odor learning and memory. In these experiments, moths were restrained in a plastic tube prior to surgery and conditioning. After surgery, a clear plastic tube was situated over the proboscis to secure a uniform position both to apply a sucrose reward (1 uL, 25% sucrose solution) and to observe maximum pumping motion and extension. Five-day-old moths were trained in a forward conditioning paradigm to associate hibiscus with the sucrose reward (**Figure 1**). The hibiscus-containing syringe was positioned approximately 5 cm from the antenna and delivered via a 5-s odor pulse. Three seconds into the pulse, sucrose was delivered to the tip of the proboscis using a pipette. This conditioning sequence was repeated six times, spaced 4 min apart. Multiple, spaced trials is a very robust form of conditioning that was employed to test shorter and longer forms of memory (Menzel, 2001).

LEARNING AND MEMORY

Proboscis extension to the conditioned odor was tested at four time points after conditioning: 5 min, 1 h, 4 h, and 24 h (**Figure 1**). These time points approximate memory traces that underlie short-term memory, short-term/intermediate-term memory, intermediate-term/long-term memory, and long-term memory, respectively (Davis, 2011).

DIURNAL TIME IN LEARNING AND MEMORY

We sought to test how an animal's physiological time of day affects learning and memory and whether the role of NO in memory is affected. To do so, we chose six time points over a 24-h period divided into photophase (day; 00:00–12:00 h) and scotophase (night; 12:00–24:00 h) (**Figure 1**). Three time points in photophase were chosen: 02:30 h, 07:30 h, and 11:30 h; and three points in scotophase were chosen: 14:30 h, 19:30 h, 23:30 h.

These time points were chosen for three main reasons: (1) 14:30 was chosen because this time approximates the hours after dusk (2.5) when *M. sexta* are actively using their olfactory systems to find mates, feed, and find sites for oviposition (Gregory, 1963–1964; Yamamoto et al., 1969). 14:30 was the time point found in the Gage et al. (2013) study that showed a robust effect of NO in short-term memory. 02:30 was used as a photophase counterpoint to examine memory 2.5 h after photophase/sunrise; (2) 11:30 and 23:30 were chosen because each preceded the light cycle switch (from photophase to scotophase and from scotophase to photophase) by 30 min, potentially illuminating an association between memory and the impending light cycle change; and (3) 07:30 and 19:30 were chosen as mid-phase time points, both 7.5 h into photophase and scotophase. When animals were in scotophase, all manipulations including the surgical manipulations and olfactory conditioning were performed under dim red light so as not to affect the circadian clock. The potential effect of the surgery itself on the circadian clock was not tested.

STATISTICAL ANALYSIS

All statistical analyses were performed using JMP 9.0.1 (SAS Institute, Cary, NC, USA). Proboscis extension reflexes were scored with a 1 or a 0 to employ parametric tests. A one-way ANOVA was performed with a *post-hoc* Tukey-Kramer HSD test. In all tests, $\alpha = 0.05$, and a 95% confidence level was used. Data are expressed as means ± s.e.m.

RESULTS

THE ROLE OF NO IN MEMORY CHANGES WITH THE TIME OF CONDITIONING

The time of olfactory conditioning influences the role of NO signaling in memory. Six conditioning times were chosen throughout the day governed by a 12 h light: 00:00–12:00/12 h dark: 12:00–24:00 cycle. At each conditioning time, animals were tested at 5 min, 1 h, 4 h, and 24 h after conditioning. What we found was a diunral, time-dependent change in the role of NO signaling in olfactory memory. **Figure 2** encompasses all six time points discussed below:

Photophase 02:30

This time point was chosen to mimic the physiological time of day 2.5 h after sunrise in light conditions. Under light conditions, or photophase, *M. sexta* are at rest. When conditioned at 02:30, L-NAME-injected animals (L-NAME is a NOS inhibitor) show a significant decrease in proboscis extension when tested at 5 min ($F_{1,67} = 7.09$, $p = 0.009$, $N = 23$), 1 h ($F_{1,67} = 18.92$, $p < 0.0001$, $N = 23$), and 4 h ($F_{1,67} = 7.61$, $p = 0.008$, $N = 23$). There was no

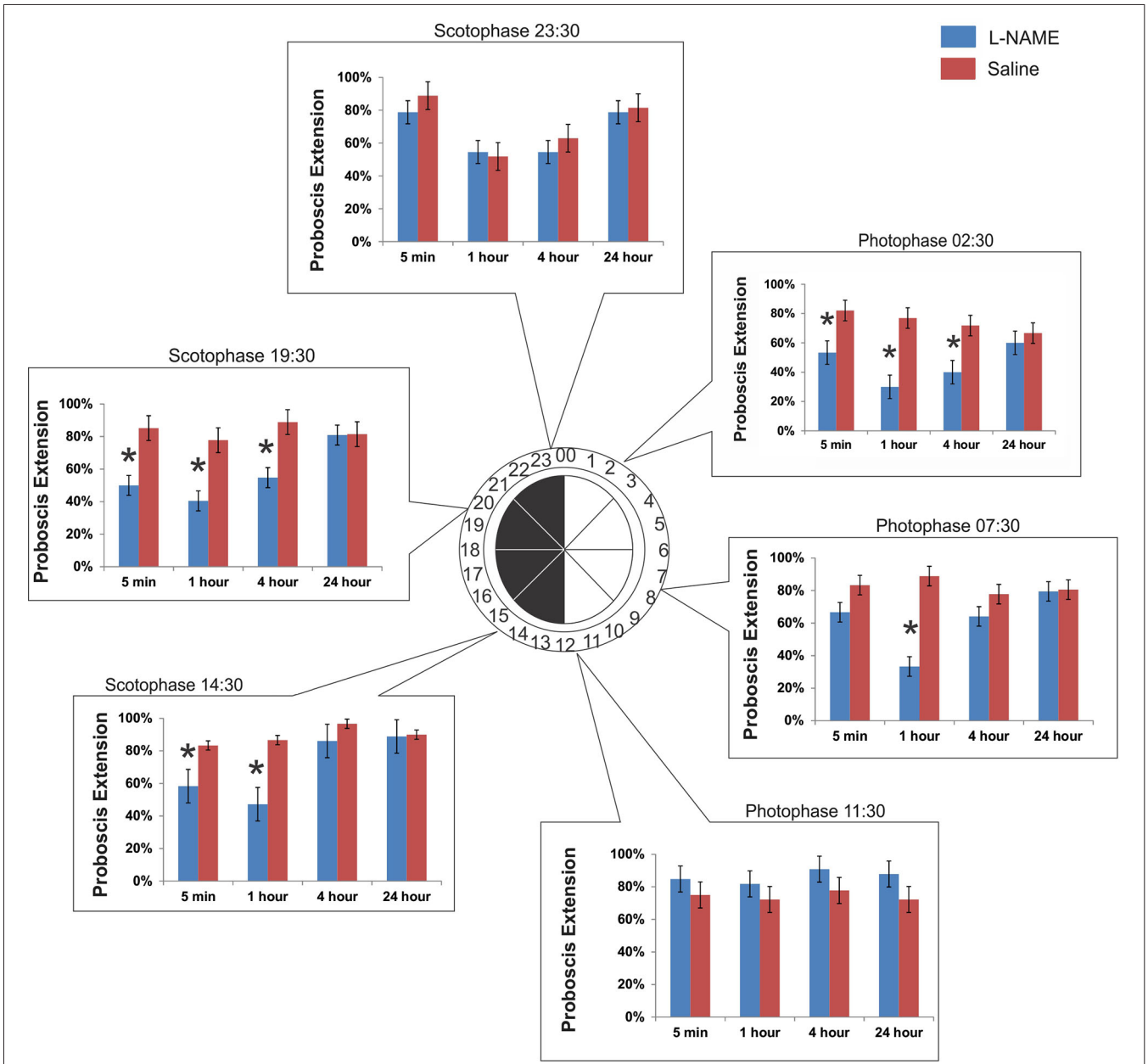

FIGURE 2 | The time of conditioning changes the effect of NO in memory. Six separate experiments were performed to test the role of diurnal time in olfactory learning and memory and the role of NO. Six time points were chosen: 3 in photophase and 3 in scotophase. Conditioning began at the start of the time point (e.g., 14:30). After conditioning, animals were tested for their response to the CS (hibiscus odor) by observing the PER at 5 min, 1 h, 4 h, and 24 h. Asterisks denote significance between treatment groups (saline-injected and L-NAME injected) using a one-way ANOVA test. Total $N = 136$.

significant effect of L-NAME *versus* saline controls at 24 h after conditioning ($F_{1,67} = 0.32$, $p = 0.57$, $N = 23$).

Photophase 07:30

This time point was chosen to assess learning and memory mid-photophase, or 7.5 h after sunrise. At this time, *M. sexta* are at rest. When conditioned at 07:30, L-NAME-injected animals show a significant decrease in proboscis extension only at 1 h ($F_{1,73} = 34.51$, $p < 0.0001$, $N = 25$). Unlike at photophase 02:30, memory tested at 5 min ($F_{1,73} = 2.78$, $p = 0.09$, $N = 25$) and 4 h ($F_{1,73} = 1.68$, $p = 0.19$, $N = 25$) did not show significant differences with saline controls. There was no significant effect of L-NAME *versus* saline controls at 24 h ($F_{1,73} = 0.01$, $p = 0.91$, $N = 25$).

Photophase 11:30

This time point mimics the physiological time of day 30 min prior to dusk and the active evening period. When conditioned at 11:30, L-NAME-injected animals are not significantly different

in proboscis extension than saline controls at any of the four time points tested (5 min: $F_{1,67} = 1.02$, $p = 0.32$, $N = 23$; 1 h: $F_{1,67} = 0.88$, $p = 0.35$, $N = 23$; 4 h: $F_{1,67} = 2.22$, $p = 0.14$, $N = 23$; 24 h: $F_{1,67} = 2.63$, $p = 0.11$, $N = 23$).

Scotophase 14:30

This time point mimics the physiological time of day 2.5 h after dusk. This time period is highly active. *M. sexta* can be found seeking mates, food, and sources to lay eggs. This time point was the time of conditioning in the Gage et al. (2013) study that reported the NO effects on short-term memory. When conditioned at 14:30, L-NAME injected animals show a significant decrease in proboscis extension at 5 min ($F_{1,64} = 5.07$, $p = 0.028$, $N = 22$) and 1 h post-conditioning ($F_{1,64} = 13.09$, $p = 0.0006$, $N = 22$). There was no significance found at 4 h ($F_{1,64} = 2.21$, $p = 0.14$, $N = 22$) or 24 h ($F_{1,64} = 0.02$, $p = 0.89$, $N = 22$).

Scotophase 19:30

This time point was chosen to assess learning and memory mid-scotophase. At this time, *M. sexta* are still active, but peak activity has begun to taper off (Gregory, 1963–1964). When conditioned at 19:30, L-NAME-injected animals show a significant decrease in proboscis extension with saline controls at 5 min ($F_{1,67} = 9.80$, $p = 0.003$, $N = 23$), 1 h ($F_{1,67} = 10.36$, $p = 0.002$, $N = 23$), and 4 h post-conditioning ($F_{1,67} = 9.81$, $p = 0.002$, $N = 23$). There was no effect of L-NAME *versus* saline controls at 24 h ($F_{1,67} = 0.003$, $p = 0.96$, $N = 23$).

Scotophase 23:30

This time point was chosen to assess learning and memory 30 min prior to sunrise. At this time, *M. sexta* are finding locations to hide and rest for the impending daytime hours. Similar to photophase 11:30 (just prior to the light switch to scotophase), L-NAME-injected animals conditioned at 23:30 do not show significant differences with saline controls at any time post-conditioning (5 min: $F_{1,58} = 1.07$, $p = 0.30$, $N = 20$; 1 h: $F_{1,58} = 0.04$, $p = 0.84$, $N = 20$; 4 h: $F_{1,58} = 0.42$, $p = 0.52$, $N = 20$; 24 h: $F_{1,58} = 0.07$, $p = 0.79$, $N = 20$).

INDIVIDUAL MEMORY WINDOWS ARE AFFECTED BY DIURNAL TIME

In addition to determining the treatment effect with L-NAME, we wanted to examine the effect of diurnal time both on the saline and L-NAME treated animals. **Figure 3** examines each of the four time points (5 min, 1 h, 4 h, and 24 h) in both saline controls and L-NAME-injected animals to determine whether conditioning time is significant.

5 min after conditioning

In this very short-term memory phase, there was no effect of diurnal time on saline injected animals ($F_{5,192} = 0.51$, $p = 0.77$). L-NAME treated moths, however, show a significant effect of conditioning time ($F_{5,204} = 2.74$, $p = 0.02$). L-NAME-injected moths appear to have a peak 5 min memory trace prior to both light cycle switches, with a trough in between time points (**Figure 2**).

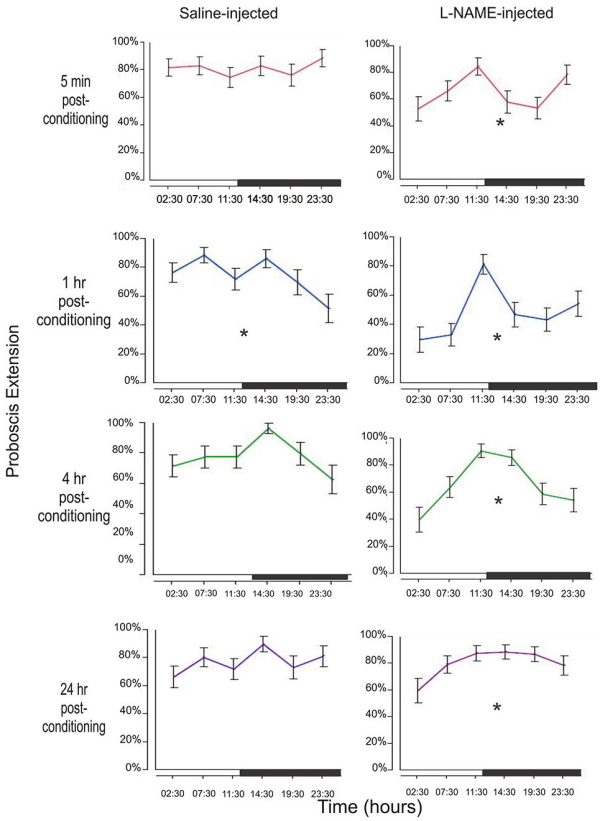

FIGURE 3 | Examination of memory by treatment and conditioning time throughout the day. Memory was tested by observing the PER to the conditioned odor (CS, hibiscus) at 5 min, 1 h, 4 h, and 24 h after conditioning. This sequence was repeated at six time points throughout the day. **Figure 3** looks at the effects of diurnal time on a single memory test (e.g., 5 min after conditioning). For example, the top left graph examines the 5 min results during each of the six time points tested around the clock. The black bar on each x-axis denotes the light cycle change (12 h light; 12 h dark) from photophase to scotophase beginning at 12:00. Asterisks denote a significant effect of conditioning time between at least one group/time point using a one-way ANOVA test. Total $N = 136$.

1 h after conditioning

This memory trace shows the most significant effect of diurnal time on the performance of control animals with a significant decrease just before the dark to light transition ($F_{5,192} = 2.99$, $p = 0.01$). L-NAME treated animals also exhibit a significant difference in proboscis extension ($F_{5,204} = 5.08$, $p = 0.0002$) with a profound change in response just before the light to dark transition.

4 h after conditioning

The 4 h time point represents intermediate-term memory with molecular mechanisms that are likely to be different from either short-term or long-term memory (Bailey et al., 2008; Berry et al., 2008; Michel et al., 2012). At this interesting time point, saline-treated animals show a borderline statistical significance between time the 14:30 and 23:30 time points ($F_{5,192} = 2.15$, $p = 0.06$, Tukey-Kramer *post-hoc* shows significance $p = 0.03$). L-NAME-treated animals also showed a significant difference in

proboscis extension among conditioning times ($F_{5,204} = 6.12$, $p < 0.0001$).

24 h after conditioning

At 24 h after conditioning, widely viewed as the time frame for long-term memory formation (Davis, 2011), the saline-injected animals do not show changes in proboscis extension among conditioning times ($F_{5,192} = 1.29$, $p = 0.27$). L-NAME-injected moths, however, surprisingly show a small but statistically significant effect of conditioning time ($F_{5,204} = 2.51$, $p = 0.03$). This finding may suggest that NO plays a small role in long-term memory as well. Perhaps under a less robust conditioning paradigm, the effects of NO in long-term memory may be seen. In addition, it may also reveal a more global effect of diurnal regulation of long-term memory. Though no difference of long-term memory was observed in the control animals, perhaps the effect at 24 h in NOS-inhibited animals underscores a masked effect.

DISCUSSION

The diurnal time of conditioning modulates olfactory learning and memory in the moth, *M. sexta*. The effect of diurnal time is subtle in control animals, with the 1 h memory trace being affected by the time of conditioning only at the dark to light transition. In animals where NO signaling is inhibited, the effect of diurnal time in memory is more significant. We find that NO can affect multiple traces of memory, and that the importance of NO signaling is modulated by diurnal time. This modulation may be attributed to interactions with other neurotransmitters and neuromodulators, like serotonin, that should also be considered for circadian contributions to memory.

NO AFFECTS LONGER MEMORY WINDOWS IN ADDITION TO SHORT-TERM MEMORY

We have previously shown that NO signaling is important for short-term memory in this olfactory conditioning paradigm (Gage et al., 2013). In this study we found that recall at the 4 h time window was affected by inhibition of NO signaling at two of the six time points tested: 19:30 and 02:30. The 4 h window reflects intermediate-term memory when compared to memory traces found in *D. melanogaster* (Davis, 2011). In *Aplysia*, this time window has been shown to be affected by both circadian time and NO in an operant conditioning paradigm (Michel et al., 2012, 2013). In that paradigm, inhibition of NOS interfered with conditioning but application of exogenous NO did not rescue the circadian-dependent inhibition. This suggests that changing NO levels could not explain the circadian-dependent effect on conditioning. The role of NO in *M. sexta* olfactory conditioning appears to be different. There is no evidence that changes in NO levels underlie the mild time-dependent effects on olfactory conditioning. Rather, the effect of NO itself on intermediate-term olfactory memory appears to be mediated by diurnal time. This reason for this effect on the role of NO is unclear, but fluctuating basal NO levels may play a role. We know that at 02:30, basal NO averages approximately 50 nM; and at peak activity time 12 h later at 14:30, basal NO is approximately 120 nM (Gage et al., 2013). These results may indicate that the effects of NO in memory formation are concentration-dependent. In moving forward, it would be helpful to determine basal NO levels at each conditioning time tested, and when 15 mM L-NAME is applied.

NO DOES NOT AFFECT MEMORY AT THE LIGHT CYCLE SWITCHES

The second intriguing finding is that inhibition of NO signaling has no effect on olfactory memory at the light cycle switches. This finding was surprising given the robust role of NOS inhibition in short-term memory. At both light cycle switches, from light to dark (11:30) and from dark to light (23:30), NOS inhibition does not produce a significant change in memory compared with the saline controls (**Figure 2**). One interpretation may be that significant physiological changes are happening to prepare for the light cycle/phase shift. The role of NO in memory may be overshadowed by other forms of neuromodulation happening here. The 11:30 time point, which proceeds the nocturnal activity period, could be especially dominated by heightened physiological activity. This activity may be modulated by several neuromodulators. The ability to form and consolidate memories is also very important at this time. *M. sexta* are especially active 1–2 h after dusk, and perhaps this crucial time is too important to be regulated by a single neurotransmitter. An interesting possibility is that serotonin levels are high enough at these times that NO modulation is not necessary. Serotonin levels vary throughout the day (Kloppenburg et al., 1999) and the single serotonergic neuron in each AL expresses sGC making it a potential target of NO (Collmann et al., 2004). It is possible that one function of NO is to increase the level of serotonin in response to odor stimulation and this effect is not necessary if serotonin levels are already high.

CONCLUSION

This study sought to shed light on two questions: (1) is there an optimal time of day for learning and memory in *M. sexta*; and (2) is the role of NO in memory modulated by the time of conditioning? In regards to the former, there does not seem to be a specific time of day in which learning and memory is optimal, but there is variation that appears phase-dependent. The role of NO in memory is also modulated by the time of conditioning. At some time points tested, NO affects longer traces of memory, in addition to short-term. There is also a curious lack of effect of NO in memory that appears specific to the light cycle switches. These unique roles of NO in memory may be the result of NO interaction with other neurotransmitters and modulators. Taken altogether, NO may be of special interest for studies examining the diurnal modulation of memory.

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Time of day influences memory formation and dCREB2 proteins in *Drosophila*

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Many biological phenomena oscillate under the control of the circadian system, exhibiting peaks and troughs of activity across the day/night cycle. In most animal models, memory formation also exhibits this property, but the underlying neuronal and molecular mechanisms remain unclear. The dCREB2 transcription factor shows circadian regulated oscillations in its activity, and has been shown to be important for both circadian biology and memory formation. We show that the time-of-day (TOD) of behavioral training affects *Drosophila* memory formation. dCREB2 exhibits complex changes in protein levels across the daytime and nighttime, and these changes in protein abundance are likely to contribute to oscillations in dCREB2 activity and TOD effects on memory formation.

Keywords: time-of-day, memory formation, dCREB2, sleep, transcription, genetic

INTRODUCTION

The daily oscillation between light and dark periods is one of the most significant changes that all animals experience. These circadian states heavily influence fundamental behaviors such as feeding, mating, and sleeping. As such, it is intuitively appealing that this system has large effects on cognitive behavior. This instinct likely results from the circadian control of sleep and the universal human experience that grogginess is not usually conducive to cognitive processes. But in most experimental contexts, this modulatory state is controlled for and does not contribute to the observed behavioral data (Gerstner et al., 2009). Circadian effects on cognition involve at least two separable processes. First, animals from *Aplysia* to humans show variable learning and memory formation capabilities across the day/night cycle (Gerstner et al., 2009). In the related observation, time stamping, animals trained at a particular time during the circadian cycle invariably perform the best when subsequently tested at that time (Holloway and Wansley, 1973). In this report, we will only discuss the variable effects of training time. However the systems, cellular, and molecular mechanisms that contribute to either aspect of this phenomenon are just beginning to emerge.

Elegant experiments using hamsters show the relationship between the circadian system and memory formation. Specific manipulation of the light/dark cycle results in animals that are behaviorally arrhythmic, but free from the pleiotropic effects that plague genetic disruptions or the anatomical confounds of tissue ablation (Ruby et al., 2008, 2013). These arrhythmic animals no longer display a circadian preference for learning/memory of a novel object recognition task. However, if GABAergic neurotransmission is blocked, the circadian effect returns, suggesting that inhibitory neurotransmission onto the hippocampus gates the performance of this task. This work demonstrates that systems-level influences are involved in circadian effects in the mammalian brain and that these processes could involve inhibition onto the areas where memory-induced changes occur.

At the molecular level, one signaling cascade important for memory formation is the ERK-MAPK pathway. This essential signaling molecule oscillates in its activity state, including in brain regions involved in memory formation (Eckel-Mahan et al., 2008). Further, long term memory expression relies on intact MAPK oscillations. Experiments that block post-training hippocampal MAPK peaks in activity inhibit memory consolidation, while inhibition during troughs in activity have no effect (Eckel-Mahan et al., 2008). These results demonstrate that naturally occurring oscillations in molecular pathways involved in memory formation are likely to be crucial for memory maintenance.

The cAMP/PKA/CREB signaling pathway is a second conserved molecular cassette that is important in both circadian and cognitive processes. In mammals, light entrainment of the circadian clock requires CREB activity (Ding et al., 1997). However, the signaling pathways leading from light presentation to CREB activation may differ depending upon the time of night. A light flash delivered during the early nighttime phase delays the circadian clock, light presentation late at night phase advances the clock, and light exposure during the middle of the night has no effect. The signaling requirements during the early and late night times differ, and one model is that cAMP/PKA serves as a molecular gate for phase shifting, allowing it to occur during

the early nighttime, but inhibiting part of the process late at night. Currently, the details of these mechanisms are unclear (Ding et al., 1997, 1998; Tischkau et al., 2000, 2003; Cao et al., 2013).

The cAMP/PKA/CREB pathway constitutes a vital component in the acute changes that occur during memory formation. Manipulating different components of the pathway can produce memory enhancement or inhibition (Kandel, 2012). Recent work suggests that the pathway also has a more chronic, sustained role in fundamental neuronal properties such as intrinsic excitability (Benito and Barco, 2010). Exactly if and how excitability influences overall memory formation remains unclear. An intriguing possibility is that a role in "(re)setting excitability" could be related to a long-term role in the maintenance of memories.

Circadian influences on learning and memory formation have also been reported in invertebrates. Early work in Aplysia showed that the time of training affects sensitization, a non-associative form of behavior, as well as associative behavior (Fernandez et al., 2003). This finding constituted a significant advance, as it reframed the question of circadian effects and moved beyond the instinctive notion that training time influences memory formation primarily through sensory gating. More recently, the circadian system has been shown to influence different behavioral paradigms and various phases of memory in Aplysia (Lyons et al., 2005, 2006; Lyons and Roman, 2008). In *Drosophila*, the time of behavioral training has been shown to affect learning, defined as the immediate acquisition and retention of associative information (Lyons and Roman, 2008). However, it has not been demonstrated in flies that the time of behavioral training can influence longer lasting forms of plasticity, including long-term memory.

The *Drosophila* dCREB2 gene has been shown to be important for circadian rhythms in flies (Belvin et al., 1999). A dCREB2- and CRE-dependent transgenic reporter (CRE-luciferase) shows an oscillatory pattern in activity, with peaks in the middle of the daytime and nighttime periods. Flies that contain a stop codon in the dCREB2 gene (S162) show a shortened circadian periodicity. Moreover, both the oscillatory pattern and the activity of the core clock Period and Timeless proteins are affected in the S162 mutant background. Finally, the oscillatory pattern of the CRE-luciferase reporter is under circadian control, corresponding to the periodicity of the fly. These results led to the conclusion that dCREB2 is part of the circadian clock, probably via transcriptional feedback loops that affect *period* expression. dCREB2 has a well established role in long-term memory formation (Yin et al., 1994, 1995a; Chen et al., 2012; Hirano et al., 2013; Tubon et al., 2013). Since dCREB2 is involved in both processes, it is logical to determine if it serves as a molecular integration point between these two systems.

In this study, we show that the TOD of behavioral training influences long-lasting memory formation. Because of the central role that dCREB2 plays in memory formation and its connections with circadian biology, we analyze dCREB2 protein isoforms across the day/night cycle. Correlations between behavior and dCREB2 protein profiles suggest that it contributes at the molecular level to TOD behavioral effects.

MATERIALS AND METHODS

ANIMALS

Fly stocks were maintained at 23°C on standard food. Experimental animals were collected 1–3 days post eclosion, housed at 100 flies per vial, and entrained to a 12:12 light:dark schedule for 3–5 days before behavioral or molecular experiments. Initial time-of-day experiments were performed with Canton S wild-type flies. Further behavioral experiments and expression experiments following behavioral training utilized the *w iso(CJ1)* strain, known colloquially as 2202U.

BIOLUMINESCENCE MEASUREMENTS

The *in vivo* luciferase assay was performed as described previously (Brandes et al., 1996; Stanewsky et al., 1997; Belvin et al., 1999; Tanenhaus et al., 2012). Briefly, flies carrying an upstream activating sequence driven flippase and a flippase-dependent CRE-luciferase reporter (UAS-FLP; CRE-F-luc) were crossed to wild type (yellow white) or GAL4 driver flies that carry a transgene to drive targeted expression of the yeast transcriptional activator GAL4 in specific cell types. The progeny were entrained through development to a 12:12 light:dark cycle and loaded individually into 96 well plates containing luciferin-fortified food. Luminescence was measured hourly using a TopCount microplate scintillation and luminescence counter (PerkinElmer). After 2 complete days, the plates were switched to constant darkness. The first 11 h were excluded from analysis to allow for initial substrate feeding. A smoothing function is applied such that each data point represents the average of three measurements.

BEHAVIORAL TRAINING

Flies were trained in the olfactory avoidance-training paradigm developed by Tully and Quinn and modified to allow for automated training sessions (described in Fropf et al., 2013). This protocol causes animals to form an association between an electric shock (the unconditioned stimulus) and a previously neutral odor (the conditioned stimulus). Each electric shock consists of 1.5 s 70-V pulses administered every 5 s over the course of 60 s (12 total). A single-cycle of training involves 90 s exposure to ambient air; 60 s of electric shock accompanied by simultaneous exposure to first odor (the conditioned stimulus condition, CS+); 45 s of ambient air exposure to clear the first odor; 60 s of exposure to the second odor, with no shock (the CS– condition), 45 s of ambient air to clear the second odor. Spaced training consists of the specified number of single cycles separated by 15-min rest intervals. 3-octanol and 4-methylcyclohexanol are used as odors.

To test memory or learning, flies are placed in a choice point and allowed to decide between the CS+ and CS− stimuli for 90 s. The performance index = [the number of flies making the correct choice] – [the number of flies making the incorrect choice]/total number of flies, multiplied by 100. To avoid odor-avoidance biases, we calculate the performance index by taking an average performance of two groups of flies, 1 trained with 3-octanol as CS+, the other with 4-methylcyclohexanol. This average of the two groups constitutes an $N = 1$.

ANTIBODIES

The antibodies used in this study and their epitopes have been previously published (Fropf et al., 2013). The αPan and αPO_4 antibodies are polyclonal antibodies raised in rabbits. The αPan antibody was raised against a sequence of amino acids in the basic region of dCREB2, and affinity purified using the antigenic peptide linked to a column. The αPO_4-specific antibody was raised against a peptide that contains the S231 residue in a phosphorylated state. Serum was passed over a peptide column containing the unphosphorylated peptide, and the flow through fraction was bound and eluted from the phospho-peptide column. The αLamin antibody used in this study is a monoclonal antibody raised in mouse, purchased from *Drosophila* Studies Hybridoma Bank (ADL67.10) and used at a 1:1000 dilution. The αHSP70 antibody is a monoclonal antibody raised in mouse purchased from Sigma (Clone BRM22, Catalog # H5147) and used at a 1:30,000 dilution.

TISSUE PREPARATION FOR WESTERN BLOT

Sample preparation procedures have been previously published (Fropf et al., 2013). Briefly, flies were flash-frozen in liquid nitrogen and heads were isolated using sieves on dry ice. To prepare nuclear and cytoplasmic fractions from collected heads, heads were homogenized on ice 3 times, for 30 s, in a homogenization buffer containing 15 mM Hepes, pH 7.5, 10 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail tablets (Roche cOmplete ULTRA Tablets, Mini, EDTA-free, EASYpack 05 892 791 001). Debris was eliminated using three 5 min 200 g spins and supernatant was collected each time for the subsequent spin. To collect nuclear sample, pellets were saved and combined from three spins at 1000 g for 5 min. Nuclear pellets were washed once with homogenization buffer and re-spun for purer nuclear samples. Cytoplasmic samples consisted of the supernatant following the third nuclear spin, which contained the carry over, non-nuclear material from the previous two spins. Protein concentrations were determined using a Bradford assay and normalized across samples in an experiment. Samples were mixed with a 2x loading buffer and boiled before loading into an SDS-PAGE gel or storing at −80°C for future use.

WESTERN BLOTTING

Samples were resolved with 12% SDS-PAGE and transferred to a Whatman Protran nitrocellulose membrane using electrophoresis. Membranes were blocked for 30 min at room temperature with milk (5% milk in 1 × Tris Bufffered Saline with Tween20 (TBST)) and incubated overnight at 4°C with primary antibodies in milk. The following antibodies were used: αPan-CREB (1:5000), αPO_4-CREB (1:1000), αLamin (1:5000), and αHSP70 (1:20,000). Following incubation in primary antibody, membranes were rinsed in TBST 4 × 15 min, blocked in milk, and incubated in secondary antibody (Jackson ImmunoResearch laboratories, Peroxidase AffiniPure Goat Anti-Rabbit IgG 111-035-003 or Goat Anti-Mouse 111-030-003) for 1 h at room temperature. After secondary antibody incubation, membranes were rinsed in TBST 4 × 15 min then incubated for 4 min in Pierce Western Blotting Substrate ECL reagent (Prod #32106). Membranes were exposed to film (Denville Scientific Premium Radiography Film, Cat #E3018) for time periods ranging from 5 s to 15 min prior to developing the film. Images of film were scanned and digitally saved using HP Scanner, version 2.4.4 (3).

During quantification, each dCREB2 band was normalized to the loading control (HSP70 for cytoplasmic samples, Lamin for nuclear samples). Values for each dCREB2 band are presented as fold change over the Zeitgeber Times (ZT0) value for that isoform. An appropriate film exposure length was selected for each isoform analyzed to ensure sufficient signal for the investigation.

STATISTICAL ANALYSIS

Behavioral data are presented as mean ± SEM. R (R Development Core Team, 2013) software was used for all statistical tests. The α-value was set to 0.05 for all assessments. For all behavioral experiments, independent two-sample *t*-tests were performed to provide between-subjects comparisons of animals trained or tested at different times during the day. To assess differences between individual and grouped time points, the Welch-Satterthwaite equation was employed to control for differences in sample size.

RESULTS

Flies that contain a CRE-luciferase transgenic reporter show that dCREB2 transcriptional activity oscillates across the day/night cycle under the control of the circadian system (Belvin et al., 1999). When this report was published, it was not possible to determine whether this pattern of activity was characteristic of certain tissue and cell populations or was widespread. A second-generation reporter was made that allowed tissue-specific expression to be imposed on the reporter (Tanenhaus et al., 2012). When the reporter is expressed in different adult head tissues and cell clusters, the same oscillatory pattern is present in all areas where signal can be detected. **Figure 1** shows the patterns of activity that are detected when reporter expression is restricted to different circadian and memory-related cell populations. Regardless of which brain cell types express the reporter, the same oscillating pattern of activity is evident. For this experiment, flies were entrained on a 12:12 light-dark schedule and CRE-luci activity in intact flies was measured for 2 days under light-dark conditions and 3 days under dark-dark conditions (the lighting conditions are indicated under the graph). As with the first generation reporter, CRE-luci activity oscillations persist into dark-dark conditions, indicating that these cell type-specific oscillations are also under circadian control. Diurnal peaks of activity are detected in sensory neurons of the visual system, integration neurons in the mushroom bodies, and in all of the different cell types of glia (**Figure 1**; Tanenhaus et al., 2012). These results lead to two predictions about dCREB2 activity. First, TOD may affect dCREB2 function in brain processes such as memory formation since most if not all brain regions show oscillations in activity. Second, since the cycling pattern is apparently ubiquitous, biochemical analysis of dCREB2 using an extract homogenized from all cells in the head could reveal part of the mechanistic bases for oscillatory activity.

In order to test TOD effects on memory formation, flies entrained to a 12:12 light:dark cycle were trained using seven

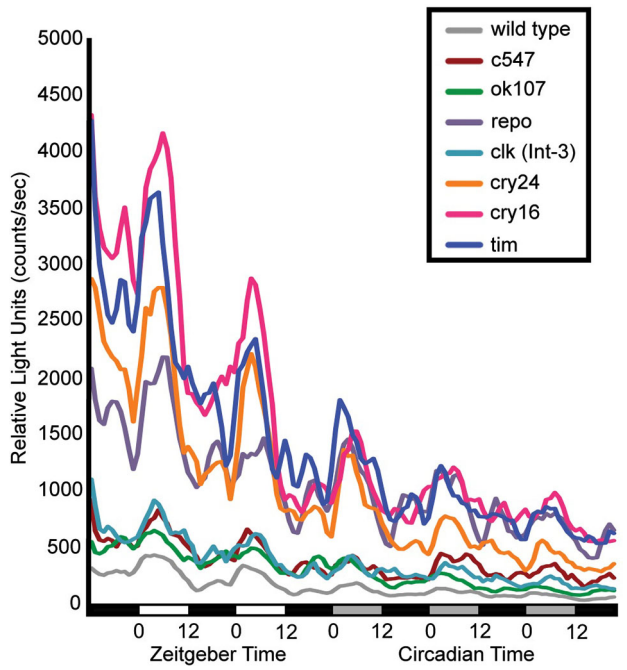

FIGURE 1 | dCREB2 transcriptional activity has a similar pattern of oscillation across cell populations. Bioluminescence is plotted in multiple fly lines expressing a dCREB2 luciferase reporter under the control of tissue-specific GAL4 drivers. Each line represents the mean reporter activity over time (hours) across flies (n = 48) representing the progeny of UAS-FLP;CRE-Fluc transgenic flies crossed with wild type (gray), or GAL4 driver lines: *c547* (red), *ok107* (green), *repo*-GAL4 (purple), *clk(int-3)-GAL4* (teal), *crypGAL4-24* (cry24, orange) *crypGAL4-16* (cry16, pink), *tim*-GAL4 (blue). Each data point represents the average over three hourly time points. Light conditions are indicated along the x-axis first in LD (black: night, white: day), then in DD (black: subjective night, gray: subjective day).

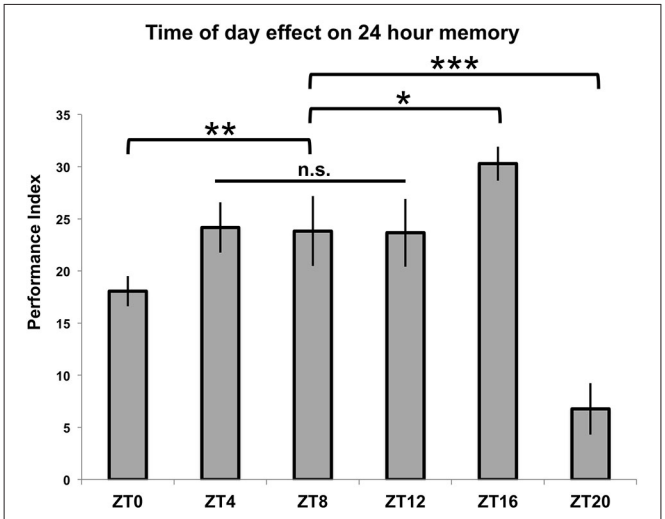

FIGURE 2 | Time of training affects 24-h memory. Wild-type animals were entrained to a 12-12 light-dark schedule, trained with 7x spaced olfactory conditioning at six different Zeitgeber Times (ZTs) throughout the day, tested 24 h after training began (n = 7–8 for all groups). Performance index values are presented as experimental means and error bars indicate SEM. Animals trained at ZT4, ZT8, and ZT12 exhibited statistically similar memory scores ($p > 0.9$ for all individual comparisons between groups) and were therefore combined to serve as a baseline memory value for other comparisons. Animals showed diminished memory scores compared to baseline when trained at ZT0 ($p < 0.01$) and ZT20 ($p < 0.001$), while animals trained at ZT16 displayed enhanced memory retention ($p < 0.05$). Statistical differences between groups are indicated as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

cycles of repetitive spaced trials which began at six different time points (ZT = 0/24, 4, 8, 12, 16 and 20). These flies were then tested for memory formation 24 h later, at the time when their training began. The results are shown in **Figure 2**. For three of the time points (ZT = 4, 8 and 12), there is no difference in performance ($p > 0.9$ for all individual comparisons between groups), and these times are subsequently treated as a baseline level of performance. When training that starts at ZT = 16 is compared to this level, there is a significant enhancement of performance ($p < 0.05$). Conversely, there is a large, significant decrease in memory scores when training begins at ZT = 20 ($p < 0.001$) as well as a smaller decrease that is still significant when training begins at ZT = 0/24 ($p < 0.01$).

To show that these differences in performance result from TOD effects on memory formation, we tested for TOD effects on learning at ZT = 16 and ZT = 20. **Figure 3A** shows that flies trained with a single training trial at ZT = 16 or ZT = 20 and tested immediately after the end of training perform similarly ($p = 0.09$), indicating that there is no TOD effect on learning under these experimental conditions. Correspondingly, flies trained with 10 (rather than 7) cycles of spaced training beginning at ZT = 16 or 20 and tested immediately after the end of training do not show any difference in performance ($p = 0.8$, **Figure 3B**). Since the immediate performance after 10 cycles of spaced training is identical whether the flies are trained at ZT = 16 or ZT = 20, it is unlikely that the major effect of the training time is through peripheral factors, such as olfactory acuity.

When different groups of flies are exposed to 10 cycles of spaced training beginning at ZT = 16 or ZT = 20 and tested for 24-h memory, there is a statistically significant difference between the two groups ($p < 0.05$), although the difference in memory scores is smaller than when flies are trained with seven spaced trials ($p < 0.0001$) (see **Figure 4A**, compare to **Figure 2**). In order to show that these effects on 24-h performance are not due to TOD effects on retrieval, flies trained at a common time (ZT = 12) and then tested for retrieval the next day at either ZT = 16 or ZT = 20 show identical performance ($p = 0.7$, **Figure 4B**). Since there are no differences in immediate performance between flies trained at ZT = 16 and ZT = 20 (**Figure 3**), and retrieval is identical (**Figure 4**), the differences in performance shown in **Figure 2** are most likely attributable to TOD effects on the consolidation steps after immediate performance but before retrieval.

One important molecule that affects the formation of long-term memory is dCREB2. dCREB2 also affects the circadian clock, which presents a logical line of enquiry: does dCREB2 also contribute to the TOD effects on memory formation? We used Western analysis of whole head extracts to assess the status of dCREB2 protein isoforms across the day/night cycle. The dCREB2 gene utilizes alternative splicing and alternative

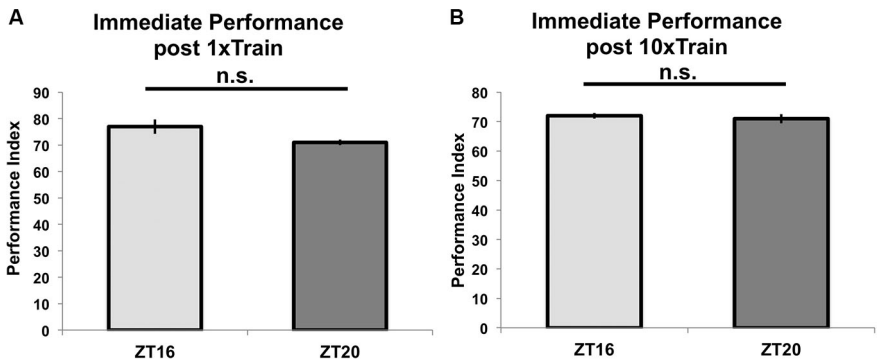

FIGURE 3 | Time of training does not affect learning or immediate memory. Wild-type flies entrained to a 12-12 light-dark schedule were trained at either ZT16 or ZT20 with **(A)** a single trial of paired olfactory conditioning or **(B)** 10x spaced olfactory conditioning ($n = 8$ for all groups). Animals were tested immediately after training, and performance is presented as experimental means with error bars representing SEM. Flies trained with a single trial at ZT16 and ZT20 show statistically similar learning scores ($p = 0.09$). Likewise, flies given 10x spaced olfactory conditioning perform similarly ($p = 0.8$) when tested immediately after training.

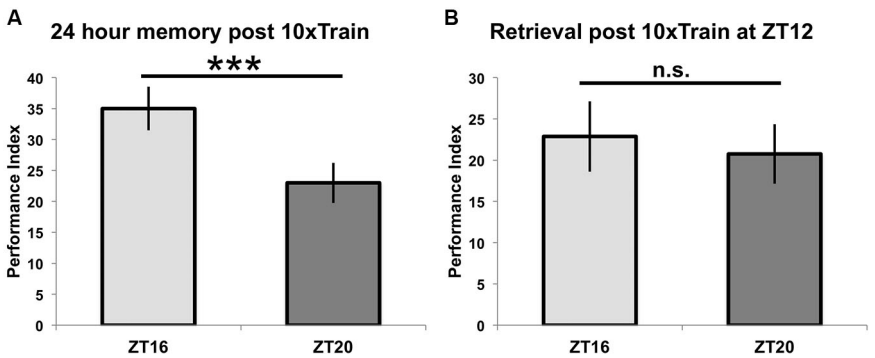

FIGURE 4 | Time of training but not time of testing affects long-term memory. (A) Wild-type animals were entrained to a 12-12 light-dark schedule and trained at either ZT16 or ZT20 with 10x spaced olfactory conditioning, then tested 24 h later ($n = 8$ for both groups). Flies trained at ZT16 showed significantly better memory ($p < 0.05$) than flies trained and tested at ZT20, although the difference between groups is less than when flies were trained with 7x spaced conditioning (compare to **Figure 2**). **(B)** After entraining to a 12-12 light-dark schedule, wild-type flies were trained at ZT12 with 10x olfactory conditioning and tested for memory retention on the following day at ZT16 or ZT20 ($n = 8$ for both groups). Flies tested at different times showed statistically similar memory retention ($p = 0.7$). Data are presented as experimental means and error bars indicate s.e.m.

translation initiation to make a large number of protein isoforms (Yin et al., 1995b; Tubon et al., 2013). These proteins in turn can be post-translationally modified in numerous ways, altering their mobility on denaturing protein gels and creating a large pool of dCREB2 isoforms which can presumably differentially affect dCREB2-mediated processes (Fropf et al., 2013).

For the sake of simplicity, we have historically grouped these different species into two functional categories, activators (A) and blockers (B). Since the activator functions in the nucleus, but most of the protein is sequestered in the cytoplasm, subcellular localization controls its function. Recently, we showed that the levels of a p35+ activator species transiently increase in the nuclear compartment after training that produces long-term memory (Fropf et al., 2013). This short-lived increase is most likely causally involved in the dCREB2-dependent events necessary for long-term memory formation. Because of these results, we examined the dCREB2 protein isoforms in the nuclear and cytoplasmic compartments across the daytime/nighttime.

Head extracts were made from flies entrained on a 12:12 light/dark cycle and collected at hourly time points. The extracts were separated into nuclear and cytoplasmic fractions and analyzed using Western blots. All 24 samples from across the day/night cycle were normalized for protein concentrations to ensure that equal amounts of protein were loaded in each lane. To confirm our equivalent protein load across lanes, the blots were cut along the 60 kD line and the upper portion of the blots were probed with a lamin- or Hsp70-specific antibody to verify roughly equal levels of nuclear or cytoplasmic protein (bottom image in each panel). Quantitating this experiment is technically very challenging, partly due to the number of samples (24) that have to be processed at one time, and partly because of the huge disparity in abundance between the normalization proteins (lamin and Hsp70) and the dCREB2 isoforms. In order to detect differences

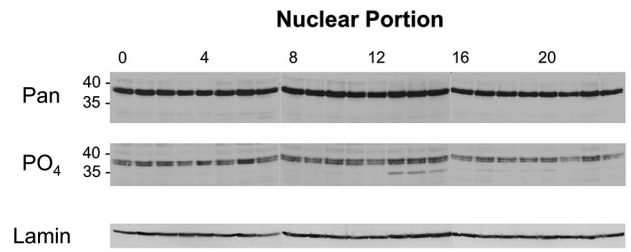

FIGURE 5 | Levels of nuclear dCREB2 protein isoforms oscillate through the day/night. Representative Western blot images of nuclear dCREB2 isoform oscillation within a 24 h period. Wild-type animals were entrained to a 12-12 light-dark schedule and flies were collected at the beginning of each hour throughout the day. Heads were isolated and then separated into nuclear and cytoplasmic fractions for subsequent analysis using SDS-PAGE and Western blot. Each set of samples was probed with an α-Pan (top) and α-PO_4 (middle) antibody to assess localization of dCREB2 isoforms across the day. Blots were probed with Lamin (bottom) as a loading control.

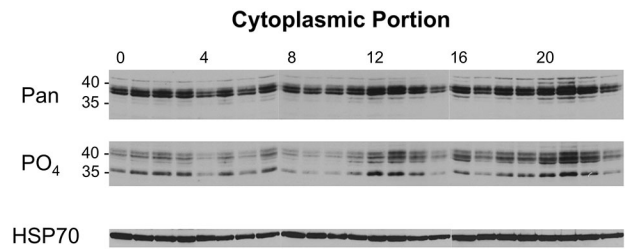

FIGURE 6 | Levels of cytoplasmic dCREB2 protein isoforms oscillate through the day/night. Representative Western blot images of cytoplasmic dCREB2 isoforms show oscillation within a 24-h period. After entraining to a 12-12 light-dark schedule, wild-type animals were collected at the beginning of each hour throughout the day, heads were separated, and cytoplasmic protein was isolated for each sample. Samples were analyzed using SDS-PAGE and Western blot and were probed with HSP-70 as a loading control. To analyze dCREB2 isoform levels across the day, cytoplasmic samples were probed with an α-Pan (top) and α-PO_4 (middle) antibody.

in the abundance of dCREB2 protein isoforms, large amounts of cytoplasmic or nuclear protein need to be analyzed.

Figure 5 (top panel) shows the Western image for nuclear samples probed with two different dCREB2 antibodies. When the blots are probed with a pan-dCREB2 antibody, the major identifiable bands are the doublet blocker species that run with an apparent molecular weight near 40 kD (top panel). There is no readily apparent large difference in the amounts of these proteins across the day/night cycle, a result that is consistent with previous data (which only sampled 6 time points) using a dCREB2-specific monoclonal antibody. However, when the blots are probed with the S231 phospho-specific antibody, there is a clear increase in the p35+ species between ZT = 13 and ZT = 15. Although a longer exposure shows that this species can be detected in nuclear samples from all time points, its levels increase when nuclear extracts are sampled during this time window.

A similar analysis was performed with the cytoplasmic samples from flies collected across the day/night cycle, and these results are presented in **Figure 6**. When these samples were probed with the pan-dCREB2 antibody, three changes occurred across the day/night cycle. First, there appears to be a slight decrease in the amount of blocker protein around ZT = 4–6. Additionally, there is a noticeable increase during the ZT = 12–14 period, and an even larger increase during the ZT=20–22 period. These changes are corroborated with the S231 phospho-specific antibody; although there are minor differences between the two antibodies, the general expression pattern is very similar.

To quantify these differences, we used ImageJ to measure the dCREB2-specific signal intensity for both dCREB2 antibodies and the corresponding loading control for each of the 24 time points. The dCREB2 signal at each time point was then normalized to its loading control, and the value at ZT = 0 is set to 1. All other time points across the daytime and nighttime are expressed relative to the ZT = 0 value. **Figure 7** shows the relative fold change for each time point plotted as a function of the zeitgeber time (ZT). Panel 7A shows the two plots for the activator species (nuclear and cytoplasmic) detected with the α-PO_4 antibody (based on the data shown in **Figure 5**), and all four plots (based on the data presented in **Figure 6**) for the blocker (either nuclear or cytoplasmic compartments probed with either the α-Pan or α-PO_4 antibody). For the activator, the most noticeable change is the abundance of the nuclear activator species (shown in solid gray in **Figure 7A**) during the ZT = 12 to ZT = 16 window. There is almost a nine-fold increase in its abundance during this period, with smaller peaks later on during the nighttime.

To the naked eye, there seems to be a late nighttime increase in the doublet that corresponds to blocker-related species (Fropf et al., 2013), but there is also a clear increase in a cluster of bands (see **Figure 6**) that are most likely to be hyperphosphorylated forms of the blocker (Zhang et al., 2013). The molecular identity of each band is unclear, since there are at least eight phosphorylation sites that are present on the dCREB2 blocker proteins, and which are conserved on all CREB family members. It is likely that combinatorial/differential phosphorylation contributes to these small differences in apparent mobility. Regardless of their molecular distinctions, this cluster of bands increases during the late nighttime, and this increase is detectable with both antibodies. **Figure 7B** shows the different quantitative patterns that are seen with both antibodies for the hyperphosphorylated and unphosphorylated or mildly phosphorylated (doublet) blocker species. The largest increase is seen with the hyperphosphorylated blocker species (dotted black trace in **Figure 7B**) detected using the αPO_4 antibody, but a qualitatively similar pattern is detectable using the α-Pan antibody (solid black trace). Consistent with these results, the doublet bands detected using the α-PO_4 antibody (dark gray in **Figure 7B**) also show a late nighttime peak. Finally, there is a general decrease in blocker and hyperphospho-blocker expression during the mid-day period, although this general effect is not as striking as the nighttime changes in abundance.

Figure 7C summarizes the dynamic changes in abundance that we believe to be most functionally important. This plot illustrates the large increase in nuclear activator (dark gray trace in **Figure 7C**) during the early evening. It also highlights

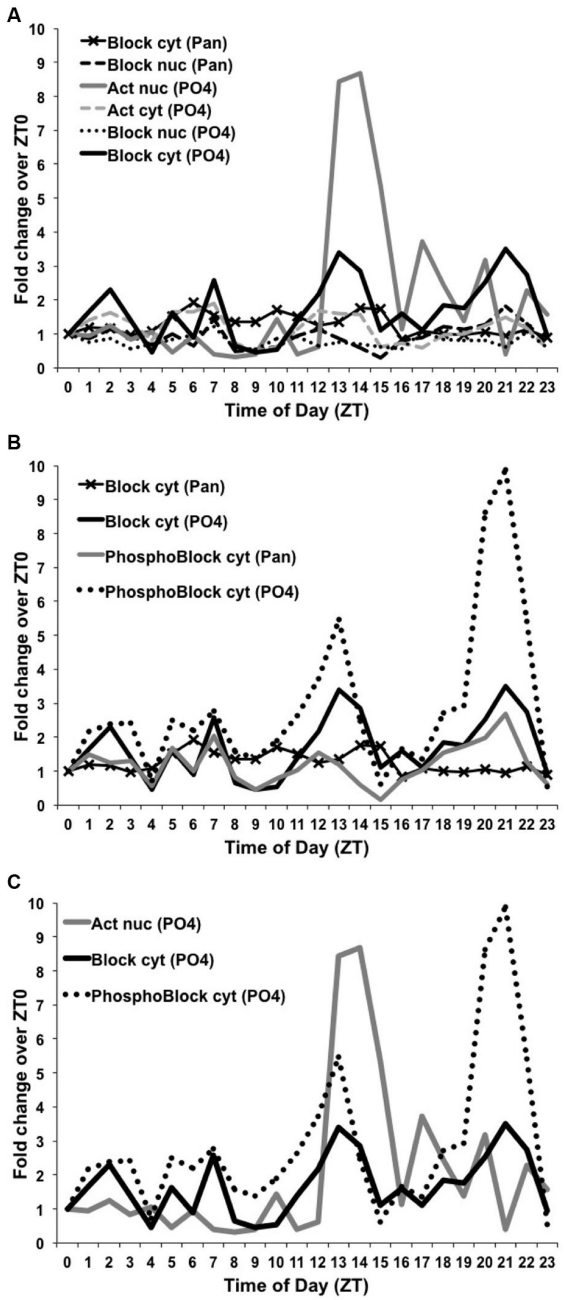

FIGURE 7 | Levels of dCREB2 protein isoforms oscillate through the day/night. Quantification of Western blot images from samples generated by wild-type flies collected each hour across a day (see **Figures 5** and **6**). Each dCREB2 band was normalized to the appropriate loading control across time points. Data are presented as fold changes of the normalized values for each isoform compared to the normalized ZT0 value for that isoform. p35 Activator, p40 Blocker, and hyperphosphorylated p40+ Blocker dCREB2 species (labeled "Act", "Block", and "PhosphoBlock", respectively) are presented for both nuclear (nuc) and cytoplasmic (cyt) samples. **(A)** Illustrates the nuclear increase in Activator during the early night (solid gray line) and fluctuating levels of cytoplasmic Blocker, which show multiple peaks and troughs (solid black line). **(B)** Highlights the strong early- and late-night peaks in cytoplasmic, hyperphosphorylated Blocker species (dotted black line). **(C)** Three dCREB2 species have time points with greater than 2-fold increases over ZT0 values.

the increases in hyperphospho-blocker (dotted black trace) and blocker (solid black trace) during the late nighttime.

DISCUSSION

Our analysis shows that dCREB2 activity oscillates (probably in all tissues) across the daytime and nighttime, and that this pattern of activity is under circadian control. In this report, we show that the TOD affects long-lasting memory formation, with a peak in performance during the early nighttime and a significant trough in the late night. Our experiments do not rigorously show that the TOD effect is under circadian control, nor that the TOD is affecting bona fide long-term memory. However, since dCREB2 activity oscillates under circadian control, and its protein isoforms show patterns of abundance that change across the day/night cycle, the simplest explanation is that the circadian-controlled oscillations in dCREB2 activity are partly responsible for the TOD effects on behavior. This would implicate TOD affecting LTM, since dCREB2 is not required for ARM, but further experiments are needed to clarify this point. Our biochemical analysis is the first report that levels of multiple dCREB2 proteins oscillate across the daytime and nighttime. These observations raise several intriguing possibilities about the molecular mechanisms underlying TOD effects on memory formation.

Previous work in invertebrate systems, including the fly, shows that olfactory processing is under circadian control, with a distinct peak in acuity that occurs during the early nighttime (Krishnan et al., 1999, 2001; Decker et al., 2007). Our behavioral experiments show a TOD memory peak during the early nighttime as well as memory troughs late at night and at the night/day transition. Is it possible that our memory effects are attributable to TOD effects on olfactory acuity? Although formally possible, we think this is unlikely. Neither single cycle training, nor repetitive spaced training, produces differences in immediate performance tested at the end of training. In neither case are the performance scores "saturated", indicating that subtle effects of olfactory acuity would most likely be measurable. In the case of repetitive spaced training, the TOD also does not affect retrieval. Therefore, it is most likely that the observed differences in performance are due to differences in molecular and cellular processes that occur after training but prior to retrieval (ie., during consolidation).

The Western analyses of dCREB2 protein isoforms suggest part of the mechanistic underpinnings of the oscillating CRE-luciferase pattern and the TOD behavioral results. Using the simple categorization of protein isoforms into activators or blockers, the heuristic activator-to-blocker ratio (A/B) is a useful preliminary means by which to correlate activity and behavior with molecular data.

The timing of the nighttime peak in CRE-luciferase activity could be attributable to the increase in nuclear levels of p35+ and the resulting increase in the A/B ratio. During the falling phase of the nighttime peak (ZT = 17–20) in activity, there is a noticeable increase in blocker-related species, which is predicted to decrease the A/B ratio. These dynamic fluctuations in protein level closely correspond to the changes in CRE-luciferase reporter activity.

Our results demonstrate notable correlations between the TOD behavioral effects and the circadian profile of dCREB2 proteins. At ZT = 20, there is a significant depression in memory

formation, an event which coincides with apparent increases in blocker-related species clearly visible on the Western blots. At ZT = 16, we measured a significant increase in performance. This time point correlates with the end of a window (ZT = 13–15) when nuclear levels of the activator are elevated. Based on these relationships, we hypothesize that the dynamics of dCREB2 protein levels contribute to the TOD effects on memory formation. A simple prediction based on these data is that optimal behavioral enhancement might occur if training begins between ZT = 13 and ZT = 15.

dCREB2 has a complex set of post-translational modifications that occur on the various protein isoforms. Our classification (activator vs. blocker) and the formulation of an A/B ratio are undoubtedly very simplistic. For example, a number of different protein isoforms probably can contribute to terms in the numerator and denominator. Similarly, the various post-translational modifications that can occur likely contribute to a specific protein's function as either an activator or a blocker. Despite this oversimplification, the use of the A/B ratio as an analytical tool produces a good correlation between the protein fluxes and the temporal peaks and troughs in activity and memory formation.

The A/B ratio can change due to increases or decreases in the numerator or denominator. It is interesting that at least two out of the four possibilities seem to occur at different points in time, suggesting that the accompanying molecular mechanisms are likely to be distinct. During the early nighttime, the A/B ratio is changed since more nuclear activator is detected, and this probably contributes to the nighttime peak in CRE-luciferase activity, and the TOD peak in memory formation. Conversely, during the late nighttime, the A/B ratio decreases because the different blocker species increase. We believe that there is a stoichiometric excess of blocker isoforms in the cytoplasmic compartment and that the majority of this protein exists as homodimeric species (B:B). Through unknown mechanisms, the blocker protein seems to be tethered in the cytoplasm. The activator proteins are much less abundant than the blockers, and this disparity might result in the majority of the activator protein existing in A:B heterodimers, which are consequently tethered in the cytoplasm. If the overall levels of the blocker proteins decrease, then the levels of A:A homodimers might increase and be able to enter the nucleus. Alternatively, A:B heterodimers might respond to signaling events and dissociate, allowing activator monomers or A:A homodimers to form and enter the nucleus. The apparent increase in nuclear p35+ during the early nighttime is most likely due to an increase in nuclear entry as described previously during memory formation (Fropf et al., 2013). However, it is formally possible that it is attributable to an increase in synthesis of the activator, or a decrease in nuclear export or degradation of the activator. Future experiments are needed to distinguish between these possibilities.

Although it is impossible at this point to distinguish amongst all of these possibilities, the important conclusion is that different underlying mechanisms seem to be employed across the day/night cycle to alter transcriptional activity. Future work will determine if the known circadian signaling pathways (cAMP, MAPK, NO, casein kinases) are employed at different times to contribute to these changes. Both the MAPK and cAMP/PKA/CREB pathways have emerged as crucial components in circadian biology and memory formation. Additionally, molecular components from both pathways show oscillatory patterns in their abundance or activity across the day/night, and these peaks and troughs correlate with, or are causally involved in, memory formation. Taken together, it is highly plausible that these and other molecules and pathways also contribute to the ebb and flow of memory formation across the day/night cycle.

Drosophila employs elaborate anatomical circuitry to process and maintain long-term memory for olfactory conditioning (Davis, 2011; Pitman et al., 2011; Wu et al., 2011; Chen et al., 2012; Pai et al., 2013). In addition to the central complex, various subdivisions of the mushroom bodies, and associated pairs of neurons such as DPM/APL/DAL, other regions or clusters of neurons are undoubtedly involved. The participation of a particular group of neurons is also likely to begin at the time of training and change during the duration of the consolidation period, which probably lasts for a few days after the end of training. This dynamic situation, in which different neurons are communicating with each other and synaptic plasticity is used to strengthen/weaken transient connectivity, probably includes both increases and decreases in dCREB2-mediated activity in different cell populations. Given the complexity of both the anatomical and molecular substrates for memory formation, it is remarkable that merely altering the initial time of training can have significant effects on such an intricate process. Although simplistic, we think the primary effects of training time on memory formation occur during or soon after the time of training, as opposed to effects during consolidation and retrieval. Since the current data with the second generation CRE-luciferase reporter suggest that most, if not all, cells in the fly head have synchronous, oscillatory dCREB2 activity, we believe that these activities, rather than ones that we cannot detect using our reporter, are responsible for the behavioral TOD effects. However, further experimentation would be needed to confirm this point.

While it may hold intuitive appeal that the worst window for memory formation occurs during the late nighttime, when flies are typically asleep, it is not as clear why the best period would also be during the night. One possibility is that other systems-level processes occur during the early nighttime sleeping period, and these processes use some of the same molecules and signaling pathways that are used for memory formation. In mammals, there is strong evidence that the different types of patterned neuronal activity (e.g., slow wave sleep, rapid eye movement sleep) contribute to memory consolidation (Walker and Stickgold, 2004; Marshall and Born, 2007). Of particular interest is the recent identification of cAMP and phospho-CREB peaks during REM sleep in mice (Luo et al., 2013). Whether fly sleep shows different electrophysiological or behavioral states is an active area of research, but there is nothing that currently distinguishes the early-from-late nighttime sleeping state (van Swinderen et al., 2004; van Alphen et al., 2013). However, it is interesting that during the late night flies sleep less and exhibit greater fragmentation of their sleep. Further technical advances are needed to explore these intriguing ideas.

Another possibility is that the peaks and troughs in memory formation could be related to circadian processes that normally occur during the nighttime and are not limited to anatomical

regions that process light. Our behavioral analysis was done under light:dark conditions (rather than in constant darkness), thus precluding a strict circadian interpretation of our results. Nonetheless, the simplest hypothesis is that circadian influences affected the TOD behavioral experiments. In mammals, phase delays during the early nighttime are known to involve the CREB pathway and result in the phosphorylation and activation of CREB without an accompanying change in the levels of unphosphorylated CREB protein (Ginty et al., 1993; Ding et al., 1997). During the late nighttime, light triggers phase advances, and these alterations in phase ultimately affect the phosphorylation state of CREB. However, the signaling pathways between light reception and CREB probably differ between the early and late night, and the resulting transcriptional effects are certainly different, since they have opposite effects on the phase of the clock. Behavioral training during the early nighttime may utilize specific signaling pathways that are normally peaking during this time, resulting in performance enhancement. Conversely, the general signaling state during the late nighttime may interfere with dCREB2 activation that is normally required for memory formation, thus resulting in poor behavioral performance when flies are trained at this time. Mechanistically linking circadian signaling with dCREB2 activity will require more knowledge about the signaling pathways used for phase delays and advances as well as the molecular events that determine the synthesis, degradation and nuclear transport of dCREB2 proteins. Regardless of the definite molecular details, our data clearly demonstrate a strong correlation between the activity of dCREB2, its role in a major neuronal function (memory formation), and its protein levels across the 24-h circadian cycle.

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CA1 hippocampal network activity changes during sleep-dependent memory consolidation

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A period of sleep over the first few hours following single-trial contextual fear conditioning (CFC) is essential for hippocampally-mediated memory consolidation. Recent studies have uncovered intracellular mechanisms required for memory formation which are affected by post-conditioning sleep and sleep deprivation. However, almost nothing is known about the circuit-level activity changes during sleep that underlie activation of these intracellular pathways. Here we continuously recorded from the CA1 region of freely-behaving mice to characterize neuronal and network activity changes occurring during active memory consolidation. C57BL/6J mice were implanted with custom stereotrode recording arrays to monitor activity of individual CA1 neurons, local field potentials (LFPs), and electromyographic activity. Sleep architecture and state-specific CA1 activity patterns were assessed during a 24 h baseline recording period, and for 24 h following either single-trial CFC or Sham conditioning. We find that consolidation of CFC is not associated with significant sleep architecture changes, but is accompanied by long-lasting increases in CA1 neuronal firing, as well as increases in delta, theta, and gamma-frequency CA1 LFP activity. These changes occurred in both sleep and wakefulness, and may drive synaptic plasticity within the hippocampus during memory formation. We also find that functional connectivity within the CA1 network, assessed through functional clustering algorithm (FCA) analysis of spike timing relationships among recorded neurons, becomes more stable during consolidation of CFC. This increase in network stability was not present following Sham conditioning, was most evident during post-CFC slow wave sleep (SWS), and was negligible during post-CFC wakefulness. Thus in the interval between encoding and recall, SWS may stabilize the hippocampal contextual fear memory (CFM) trace by promoting CA1 network stability.

Keywords: synaptic plasticity, fear memory, neural network, consolidation, hippocampus, extracellular recording, slow wave sleep, REM sleep

INTRODUCTION

Sleep plays an essential role in promoting various forms of memory consolidation (Aton et al., 2009b) and plasticity in brain circuits *in vivo* (Aton et al., 2009a, 2013, 2014; Seibt et al., 2012). Recent work has taken advantage of single-trial training paradigms to assess sleep effects on memory processes dependent on circuit plasticity (Aton et al., 2014). An example is single-trial contextual fear conditioning (CFC) in mice (placement in a novel context, followed by foot shock). Such conditioning results in a long-lasting fearful memory, measured as contextual freezing behavior upon return to the CFC context. In this paradigm, sleep within the first 5 h following CFC is an absolute requirement for long-term contextual fear memory (CFM) consolidation (Graves et al., 2003; McDermott et al., 2003; Vecsey et al., 2009; Prince et al., 2014).

Recent work has been aimed at understanding the relationship between sleep and the intracellular events required for both synaptic plasticity and memory formation. The cellular mechanisms underlying CFM *in vivo* are also critical for long-term potentiation (LTP) of CA1 hippocampal synapses *in vitro*. CFM consolidation requires both CA1 network activity (Daumas et al., 2005), and the activation of kinase and protein synthesis pathways (Bourtchouladze et al., 1998; Lattal and Abel, 2004; Sindreu et al., 2007), in the hours immediately following CFC. Because these pathways are also required for CA1 LTP, one possibility is that sleep interferes with CFM consolidation by disrupting synaptic potentiation in CA1. Recent studies have clarified intracellular events in the hippocampus during sleep vs. sleep loss (Vecsey et al., 2012) and have defined how intracellular signaling pathways are altered by sleep deprivation to impair CFM (Vecsey et al., 2009). The pathways disrupted in the hippocampus by sleep loss (e.g., mTOR-mediated activation of protein synthesis, kinase-mediated protein phosphorylation) are essential for CA1 LTP (Vecsey et al., 2009, 2012), and critically, sleep deprivation itself

interferes with CA1 LTP (McDermott et al., 2003; Kopp et al., 2006).

In contrast to our increasing understanding of the cellular and molecular effects of sleep and sleep deprivation in the hippocampus, almost nothing is known about how the hippocampal network activity changes unique to sleep contribute to memory consolidation. Two hippocampal network oscillations—theta (4–12 Hz) and sharp-wave/ripple (150–200 Hz) events—are hypothesized to promote episodic memory consolidation (Wetzel et al., 1977; Eschenko and Sara, 2008; Girardeau et al., 2009; Popa et al., 2010). These oscillations occur most prominently during rapid eye movement (REM) sleep and slow wave sleep (SWS), respectively. However, very little is known about the role of such oscillations in sleep-dependent memory consolidation. Even less is known about whether activity changes among individual hippocampal neurons occur during memory consolidation. Recent *in vitro* studies suggest that membrane excitability increases in CA1 neurons follow initial learning (McKay et al., 2009, 2013), and that long-term (i.e., 72 h) sleep deprivation reduces membrane excitability (McDermott et al., 2003). However, it remains unclear how such changes are expressed *in vivo*, or what role sleep might play in regulating *in vivo* activity changes.

To clarify how hippocampal network activity changes *in vivo* during active memory consolidation, we carried out continuous stereotrode recording of CA1 neuronal firing and local field potential (LFP) activity in mice. Recordings spanned a 24 h baseline period and for 24 h following either single-trial CFC or Sham conditioning (placement in a novel context without associated foot shock; a control for behavioral procedures not associated with CFM). We assessed how neuronal and network activity in CA1 was altered as a function of behavioral state and active CFM consolidation. Specifically, we quantified state-specific changes in CA1 neurons' firing rates and power spectral density in CA1 LFPs following either CFC or Sham conditioning. We also assessed changes in the temporal dynamics of functional communication between CA1 neurons in the hippocampal network as a function of CFC and behavioral state. By clarifying the network-level activity changes associated with consolidation of CFM, we hope to shed light on other CA1-mediated functions, such as object recognition memory (Clarke et al., 2010), spatial contextual memory (Dupret et al., 2010; Barbosa et al., 2012), and spatial representation (Henriksen et al., 2010).

MATERIALS AND METHODS

MOUSE HANDLING, SURGICAL PROCEDURES, AND DRIVEABLE HEADSTAGE PLACEMENT

All animal husbandry and surgical/experimental procedures were approved by the University of Michigan UCUCA board for animal care and use. Throughout all experimental procedures, mice were kept on a 12 h:12 h light:dark cycle (lights on at 8 AM), and were given food and water *ad lib*.

At age 2–6 months, male C57BL/6J mice (Jackson) were implanted with custom-built, driveable headstages under isoflurane anesthesia, using previously-described techniques (Aton et al., 2013, 2014). Each driveable headstage was composed of two bundles (each approximately 200 μm in diameter, spaced 1–1.5 mm apart) of 7 stereotrodes each (25 μm nichrome wire, California Fine Wire) wired onto Neuralynx electrode interface boards (EIB-36, Neuralynx). During surgical placement, stereotrode bundles were placed within right-hemisphere CA1. Reference and ground electrodes (silver-plated copper wire, Alpha Wire) were placed over left-hemisphere hippocampus and cortex, and 3 EMG electrodes were placed deep in the nuchal muscle.

RECORDING PROCEDURES

Chronic stereotrode recording was carried out using general procedures described previously (Aton et al., 2013, 2014). After 1 week of postoperative recovery, mice housed in their home cages were placed within a sound-attenuated sleep-recording chamber (Med Associates), and headstages were connected to a lightweight cable to record neural signals. Over a 3–5 day period, mice were habituated to the recording chamber and were handled daily for at least 10 min. During this time, stereotrode bundles were slowly advanced into the hippocampus in 10–20 μm steps, until stable recordings were obtained (indicated by continuous presence of spike waveforms on channels for at least 24 h). Following this period of habituation and electrode advancement, all experiments began with a 24 h baseline recording period, starting at lights-on. Signals from each electrode were split and differentially filtered to obtain spike data (200 Hz–8 kHz) and local field potential/electroencephalographic data (LFP/EEG; 0.5 Hz–200 Hz) at each recording site. Data were amplified at 20×, digitized, further digitally amplified at 20–100×, and recorded using Plexon Omniplex hardware and software (Plexon Inc.; Dallas, TX).

LESIONING AND LAMINAR ANALYSIS OF RECORDING SITES

At the end of experimental recording procedures, mice were anesthetized with isoflurane and all electrode sites were lesioned (2 mA, 3 s per wire), after which mice were euthanized and perfused with formalin. To verify CA1 electrode placement, the hippocampus was post-fixed and sectioned at 50 μm for cresyl violet staining and reconstruction of stereotrode bundle tracts and recording site lesions, using previously described procedures (Aton et al., 2013, 2014).

CFC

Following 24 h baseline recording (within 1 h of lights-on), mice underwent either CFC (Graves et al., 2003) or Sham conditioning (n = 6 per group). Mice were placed in a novel conditioning chamber with walls made of clear Plexiglas and a shock grid floor. Chamber walls and floor were cleaned with 70% ethanol both prior to and immediately following conditioning. Mice were placed into the chamber and allowed to explore freely for either 150 s (CFC mice) or 180 s (Sham mice). CFC mice then received a 2 s foot shock (0.75 mA; administered via a Med Associates Aversive Stimulator/Scrambler), and were left in the conditioning chamber for an additional 28 s. Throughout these procedures, mice were continuously video monitored using Plexon Cineplex software. Following conditioning, mice were returned to their home cage in the sleep-recording chamber and underwent an additional 24 h period of undisturbed recording prior to contextual fear behavioral assessment.

Twenty-four hour following contextual fear or sham conditioning, mice were returned to the conditioning chamber for 5 min, during which behavior was continuously video monitored. Freezing behavior (and CFM) was subsequently assessed from video recordings using previously-described methods (Vecsey et al., 2009). To quantify CFM in each mouse, context-specific freezing was quantified as a change in the percentage of total recording time spent in stereotyped freezing behavior between the 5 min test period and the pre-shock interval in the initial training period (i.e., % time spent freezing at test—% time spent freezing at baseline; **Figure 1A**).

SLEEP/WAKE, FIRING RATE, AND LFP ANALYSIS

Intrahippocampal LFP and nuchal EMG signals were used to assign polysomnographic data into periods of REM sleep, SWS, and waking states over 10 s intervals using custom software. The proportion of time spent in REM, SWS, and waking (and mean bout duration for each state) was calculated during the baseline and post-conditioning recording periods using standard conventions (Aton et al., 2013).

Single-neuron data were discriminated offline using standard principle-component based procedures (Offline Sorter; Plexon). Individual neurons were tracked throughout each experiment on the basis of spike waveform, relative spike amplitude on the two stereotrode recording wires, relative positioning of spike waveform clusters in three-dimensional principal component space, and neuronal subclass (e.g., FS vs. principal) (Aton et al., 2013, 2014). Only those neurons that were verifiably discriminated and continuously recorded throughout each experiment (i.e., those that were stably recorded across 24 h baseline and 24 h post-conditioning recording) were included in analyses of ongoing network activity. An example of representative spike data from two neurons recorded on a single stereotrode is shown in **Figure 2A** at different timepoints across baseline and post-conditioning recording periods.

For each reliably-discriminated neuron (n = 44 for Sham conditioned mice, n = 52 for CFC mice), mean firing rates were calculated separately within each behavioral state (REM, SWS, and wakefulness) across the 24 h baseline period and 24 h post-conditioning period. Mean firing rates in each state were then calculated in 1 h and 6 h windows across each recording period, as shown in **Figures 3A** and **3C**, respectively. Firing rate changes after conditioning were then expressed for each neuron as a % change from baseline in each 6 h window, as shown in **Figure 3D**. For analysis of direction of firing rate changes among individual neurons (recorded within an individual experiment, or across all experiments (left and right, respectively, **Figure 3B**)), increases and decreases in firing rate were estimated as those changes >5% from baseline. Neurons expressing an increase or decrease of ≤5% from baseline were categorized as having no change in firing rate.

Raw LFP power was calculated on each channel where spike data was stably recorded throughout the experiment. Power spectral density (PSD) values were quantified in 0.4 Hz bands, and average power in each band was assessed separately within each behavioral state (REM, SWS, and wakefulness) across 6 h windows. Changes in power at each frequency band were quantified from raw LFP traces as a % change from baseline within each

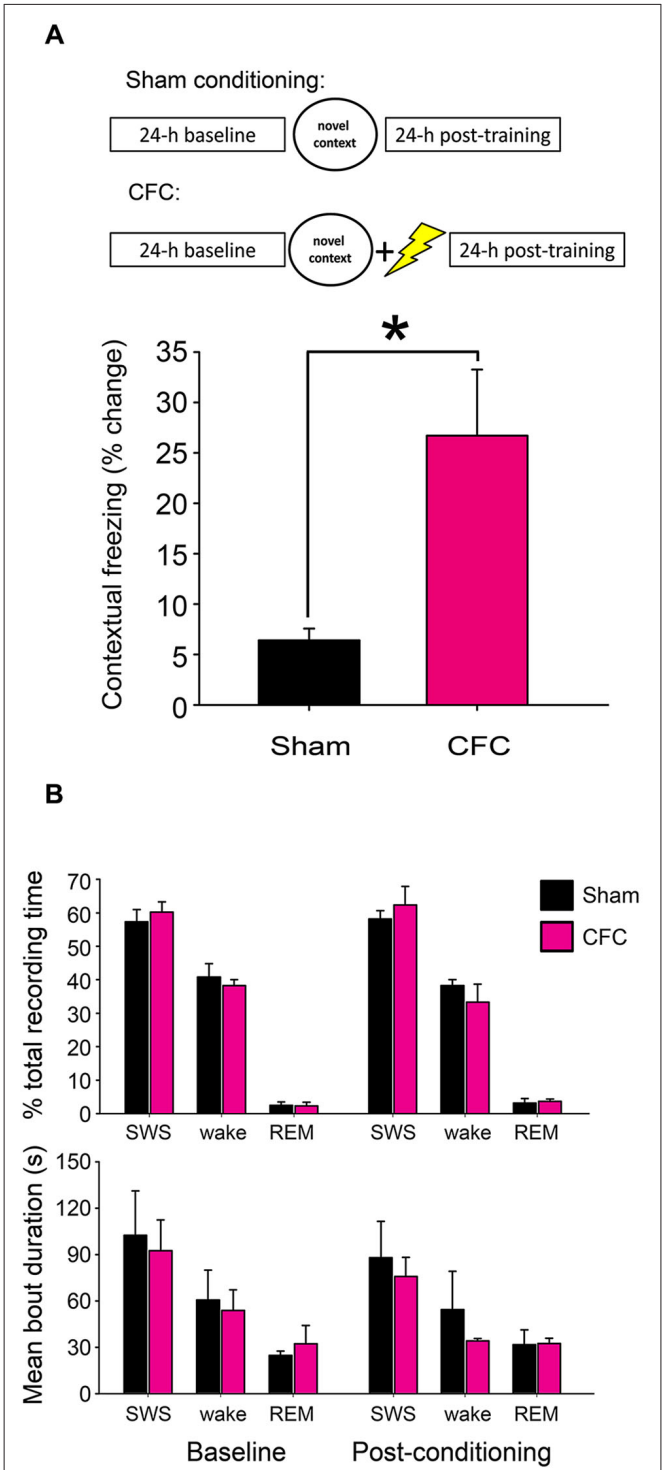

FIGURE 1 | CFC initiates fear memory formation without significantly altering sleep-wake behavior. (A) Experimental overview and quantification of context-specific freezing in Sham and CFC mice (n = 6 per group). * indicates $p < 0.05$, Student's t-test. **(B)** Sleep architecture in the 24 h following CFC was not significantly altered, either relative to pre-CFC baseline, or relative to the 24 h following Sham conditioning. Effects of group (Sham vs. CFC) and time relative to training (baseline vs. post-conditioning) on % total recording time or bout duration for each state, *N.S.*, two-way RM ANOVA.

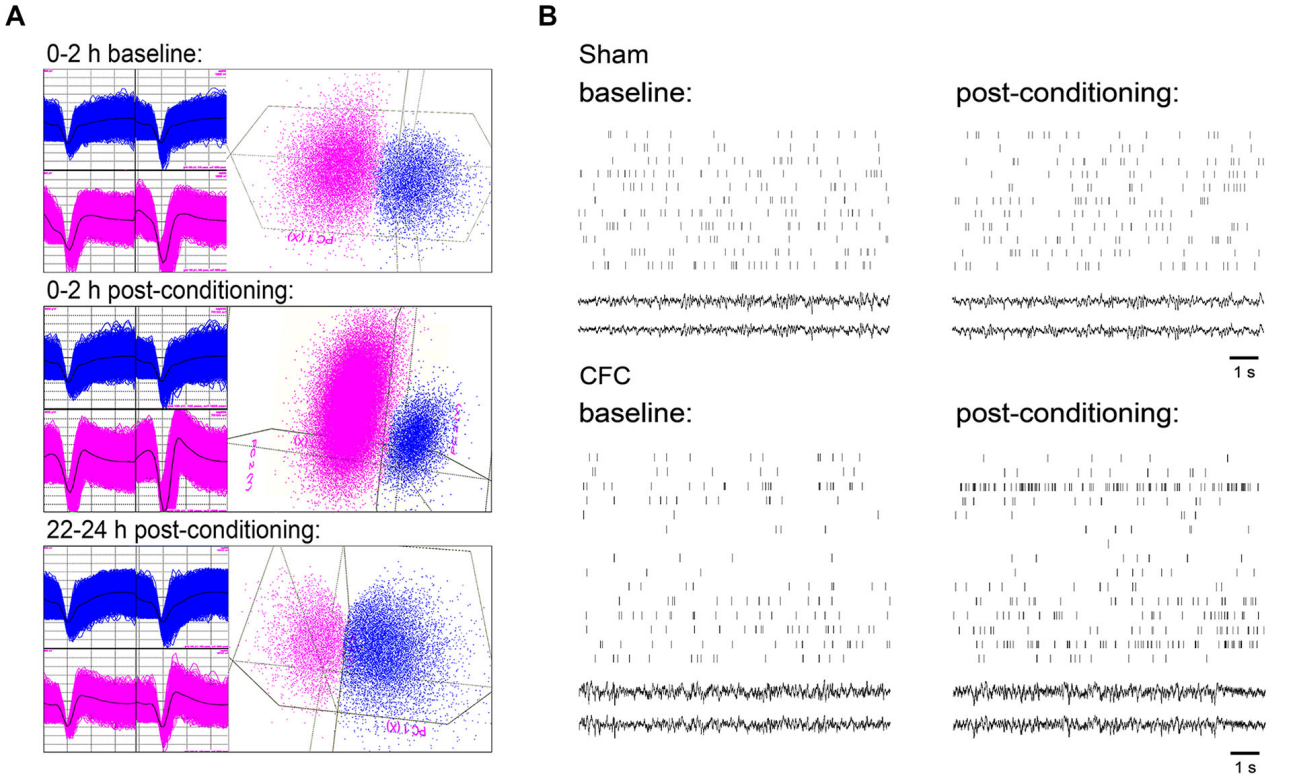

FIGURE 2 | CA1 neuronal spiking and field potential recordings. (A) Representative spike waveforms for two CA1 neurons which were continuously recorded on a single stereotrode and reliably discriminated across 24 h baseline and 24 h post-CFC periods (left), and mapping of spike waveform clusters in three-dimensional principal component space (right). Data shown comprise waveforms collected over 2 h windows, either 24 h prior to CFC (top), immediately following CFC (middle), or immediately prior to contextual fear assessment (bottom). **(B)** 10 s rasters of firing for all CA1 neurons stably recorded from a representative mouse in Sham and CFC conditions (and corresponding LFPs). Rasters are taken from intervals of SWS within the first hour of both the baseline and post-conditioning periods.

window (as shown in **Figure 4A**). For quantitative analysis of LFP changes, changes from baseline were summed across the following frequency bands for each mouse: delta (0.5–4 Hz), theta (4–12 Hz (and for comparison, also in a narrower 4–7 Hz band)), and gamma (25–50 Hz). Values shown in **Figure 4B** represent the arithmetic sum of % changes for each 0.4 Hz frequency measure across each respective band, $\times 10^3$.

FUNCTIONAL CLUSTERING ALGORITHM AND NETWORK STABILITY ANALYSIS

To assess dynamic network reorganization in the hippocampus following conditioning, and as a function of behavioral state, we used a functional clustering algorithm (FCA; Feldt et al., 2009) to assess functional network structure based on firing patterns among CA1 neurons. The FCA is a reduction-based algorithm, in which pairwise correlations between neurons in a network are calculated and the most strongly correlated pairs are iteratively merged together into a single spike train. Those trains consisting of progressively merged cells' activity are progressively joined, forming clusters of cells exhibiting similar spatio-temporal activity patterns over time. The clustering stops when the most highly correlated pair among the remaining spike trains no longer exceeds a set threshold. These correlations were calculated based on the average minimum time-distance (AMD) between the spikes of the two trains (with shorter times indicating greater correlation), normalized with reference to uncorrelated (Poisson) spike trains with the same firing rate. This normalization removes the frequency-dependence of the average minimum time metric and allows comparisons to be made between cells with distinct firing rates. FCA combined with the normalized AMD measure was used to identify number of functional clusters and identities of neurons belonging to given cluster. These interdependencies characterize the functional connectivity structure among recorded CA1 neurons and are depicted as dendrograms for given time interval (**Figure 5A**). The data was divided into 1 min bins and dendrograms were generated for the entire population of stably recorded CA1 neurons across each 1 min recording interval. Dendrogram joining values were assigned to each pair. The joining values were defined as $J_{ij} = -\log\left({}^{AMD}{}_{ij}\Big/_{P_{ij}}\right)$, where AMD_{ij} is the average minimal distance between i-th and j-th train and P_{ij} its theoretical value obtained from Poisson spike distributions having equivalent numbers of spikes. Based on these dendrograms, we established a connectivity vector for every neuron.

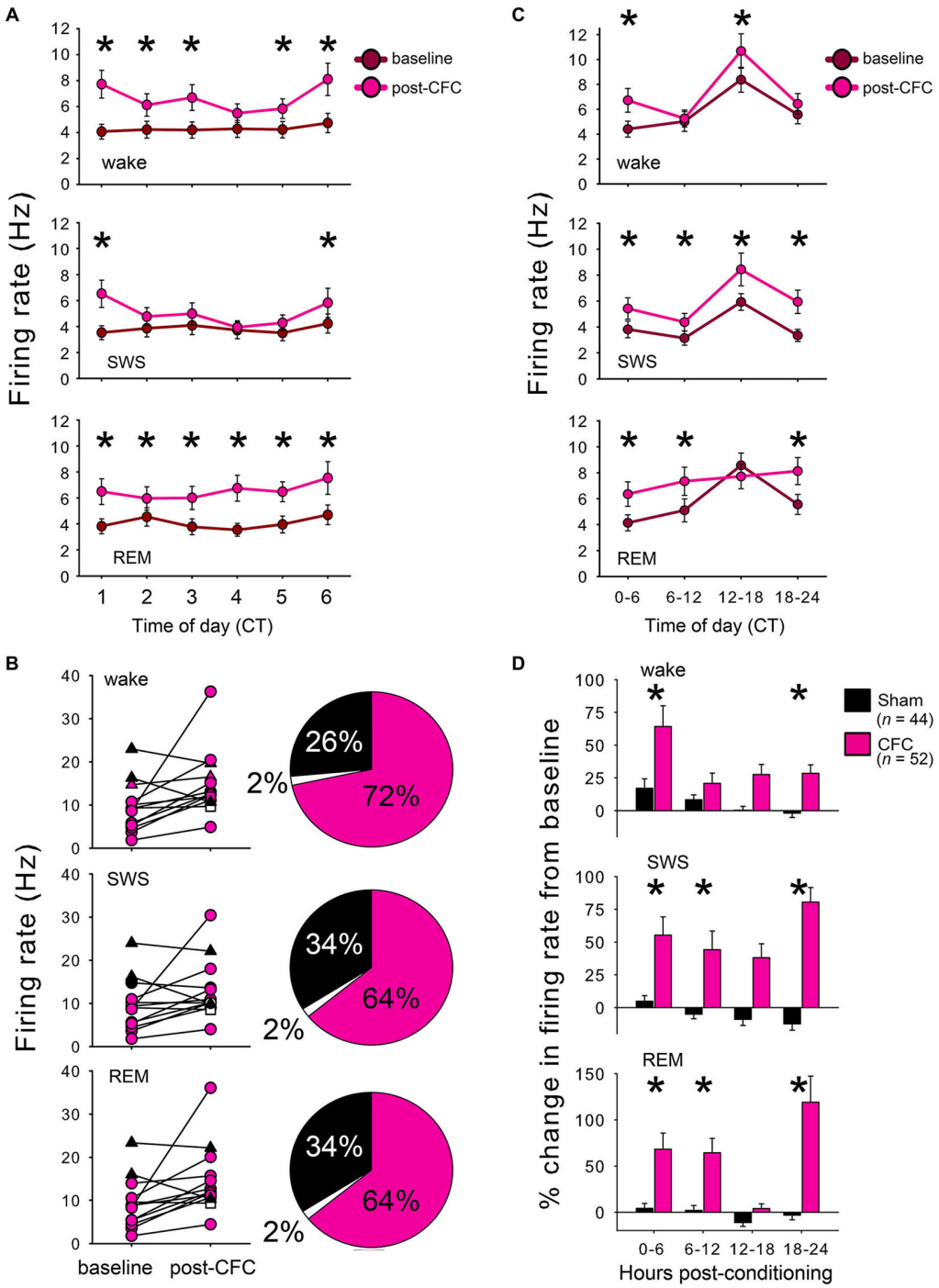

FIGURE 3 | CFC induces long-lasting increases in CA1 neuronal firing rates. (A) Mean firing rates for CA1 neurons increased within the first hours following CFC. For all 3 states, main effect of conditioning $p < 0.05$; conditioning × time interaction $p < 0.001$ for wake and SWS, two-way RM ANOVA. **(B)** Firing rate changes in each state are shown for individual neurons within a single representative CFC mouse (left) and the proportion of neurons showing increases, decreases, or no change in firing after CFC are summarized across all experiments (right). Neurons with a >5% increase in firing from baseline are shown in pink, those with >5% decrease are shown in black, and those with no change are shown in white. **(C)** CA1 neuronal firing remained elevated across the 24 h post-CFC recording period. For all 3 states, main effect of conditioning $p < 0.05$; conditioning × time-of-day interaction $p < 0.005$, two-way RM ANOVA. **(D)** CFC and Sham conditioning differentially affected firing rate. Post-conditioning firing rates for each neuron were expressed as a % change from the corresponding period of baseline recording. Effects of group (Sham vs. CFC) and time relative to training (baseline vs. post-conditioning) $p < 0.001$ for each state, two-way RM ANOVA. * indicates $p < 0.05$, Holm-Sidak *post-hoc* test for baseline vs. post-CFC comparisons.

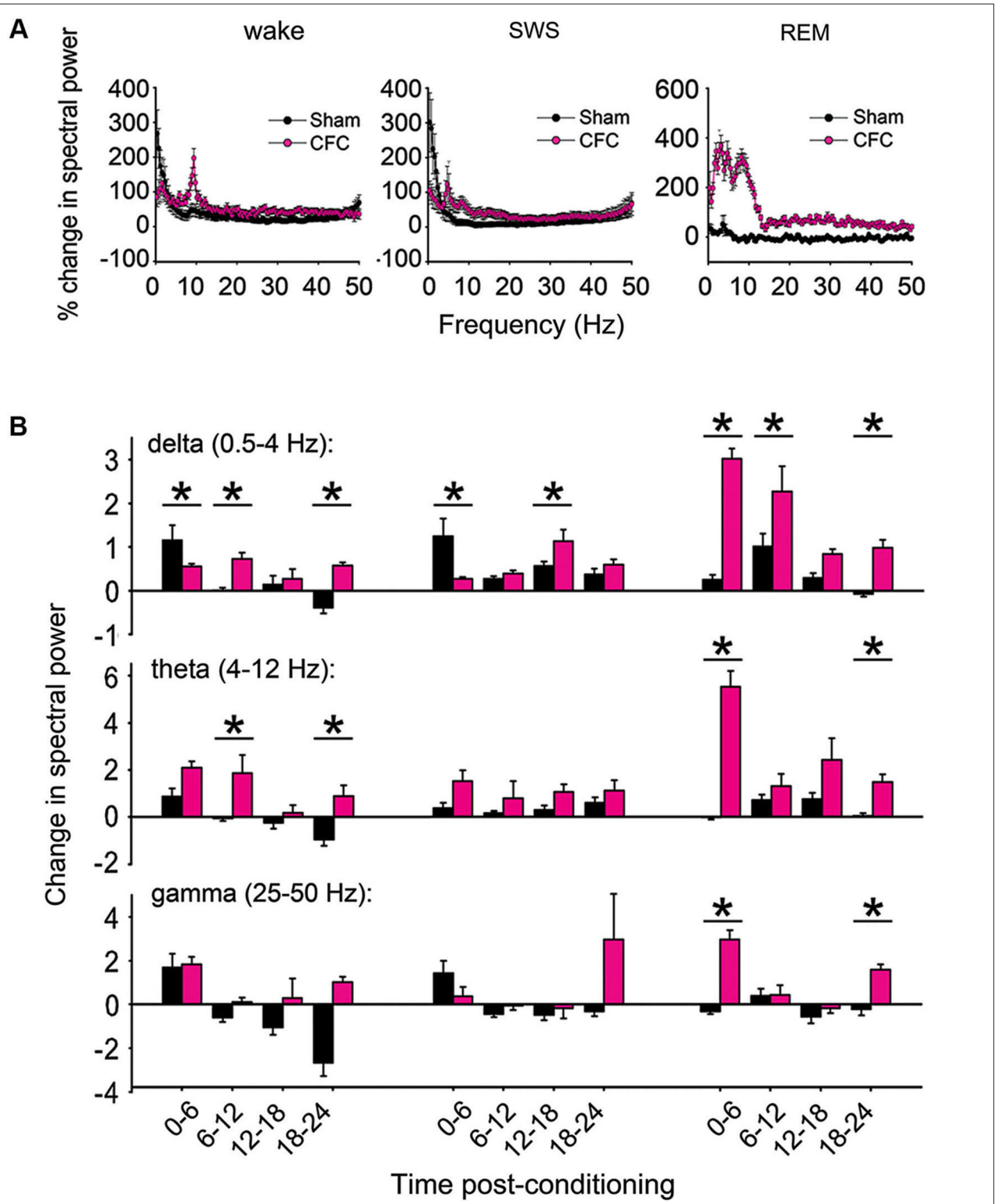

FIGURE 4 | CFC induces long-lasting increases in CA1 LFP oscillatory activity. (A) Changes in spectral power (from baseline to post-conditioning recording periods) are shown from 0–50 Hz over the first 6 h post-conditioning in wakefulness, SWS, and REM. **(B)** % changes in spectral power were summed across delta, theta, and gamma-frequency bands to compare band-specific changes following consolidation. Values represent the arithmetic sum of these changes (measured at 0.4 Hz intervals) $\times 10^3$. While the largest post-CFC changes were seen in the theta-frequency range during REM (with relative increases peaking at around 7–4 Hz), significant increases in theta were also present following CFC in wakefulness, as in delta and (to a lesser extent) gamma-frequency bands. * indicates $p < 0.05$, Holm-Sidak *post-hoc* test for baseline vs. post-CFC comparisons.

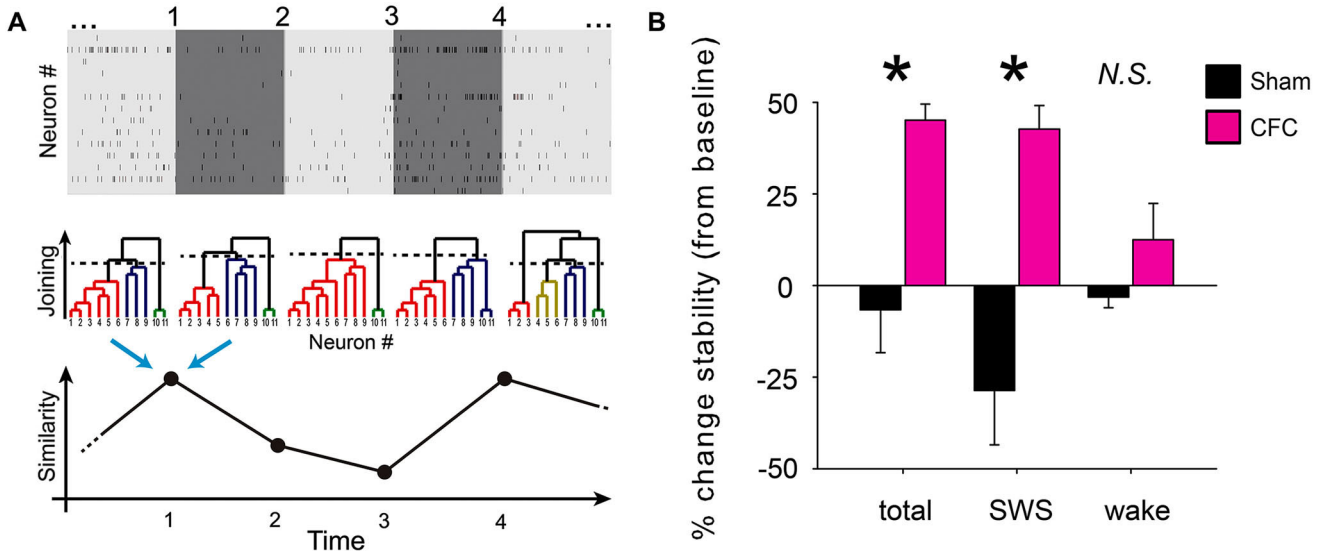

FIGURE 5 | CA1 network communication becomes more stable during SWS following CFC. (A) Schematic depiction of functional clustering algorithm (FCA) and subsequent network stability analysis. Recorded spike trains are divided into adjoining 1 min intervals (top). FCA is performed on every 1 min interval (middle) to assess functional connectivity among the recorded neurons. This analysis yields a dendrogram of network functional structure at each interval. The stability of network connectivity is established from the evaluation of the similarity of neuronal functional clustering between consecutive intervals (bottom). **(B)** Minute-to-minute network stability was averaged across baseline to post-conditioning periods. Post-conditioning changes in average stability (from baseline) are shown for Sham and CFC mice. While significant differences in stability changes were seen between the two groups when analysis was carried out across all states (total) or across intervals occurring in SWS, no significant stability differences seen for analysis over intervals of wakefulness (wake). * indicates $p < 0.01$ and $p < 0.005$ respectively for all-state and SWS-specific analysis, Student's *t*-test.

To assess whether CFC resulted in a lasting, stable change in CA1 functional connectivity, we next compared the connectivity matrices from adjacent 1 min time intervals using cosine similarity. This similarity analysis yielded values between −1 and 1, indicating the level of similarity between dendrogram structures from adjacent 1 min recording intervals, with a value of 1 indicating that the functional connectivity of the cells did not change in any way (i.e., the cells in the two time intervals formed exactly the same functional clusters with exactly the same joining distances). Thus the metric returns the degree of similarity between two adjacent dendrograms, but does not elucidate the scope of their changes (e.g., changes in the number of clusters, neuronal identities assigned to a given cluster, joining distances, etc.). The resulting minute-to-minute similarity values were averaged over the entire 24 h duration of baseline and post-conditioning recording. Changes in average stability between the baseline and post-conditioning periods were calculated separately using data from: (1) all behavioral states; (2) from epochs of SWS only; or (3) from epochs of wakefulness only (**Figure 5B**). Due to the relative infrequency and short duration of REM epochs (which typically lasted less than 1 min each) there was an insufficient number of successive recording epochs to reliably calculate network stability changes specifically within REM epochs.

RESULTS

CFC INDUCES CFM WITHOUT ALTERING SLEEP BEHAVIOR

CFM was assessed using previously described methods (Vecsey et al., 2009). As anticipated, mice receiving a foot shock in the context of exploring a novel test chamber (CFC) showed significantly increased freezing behavior when returned to the same environment 24 h later ($p < 0.05$ for CFC vs. Sham, Student's *t*-test; **Figure 1A**). While CFM consolidation is known to require sleep behavior, neither CFC nor Sham conditioning caused significant changes in subsequent sleep architecture (i.e., % of time spent in SWS, REM, or wakefulness, or mean duration of SWS, REM or wakefulness bouts) from the baseline recording period (**Figure 1B**). Two-way repeated-measures ANOVA found no significant effect of either group (Sham vs. CFC) or time relative to training (baseline vs. post-conditioning) on sleep architecture measures. Thus the role of sleep in promoting CFM consolidation is not associated with either overall increases in sleep time, alterations in SWS: REM sleep time ratio, or greater sleep continuity following CFC.

CFM CONSOLIDATION IS ASSOCIATED WITH INCREASED CA1 NEURONAL ACTIVITY

To assess changes in neuronal activity associated with CFM consolidation, firing patterns of individual CA1 hippocampal neurons were tracked continuously over the 24 h baseline recording period and for 24 h following conditioning. Firing rate changes associated with Sham conditioning and CFC were assessed among those neurons stably recorded across the entire baseline and post-conditioning periods (from which representative example data is shown in **Figures 2A–B**). CA1 firing rates for CFC mice increased significantly in all behavioral states within the first hours following CFC (**Figure 3A**; main effect of conditioning

$p < 0.005$, $p < 0.001$, and $p < 0.05$ for wake, REM, and SWS, respectively; conditioning × time interaction $p < 0.001$, *N.S.*, and $p < 0.001$ for wake, REM, and SWS, respectively, two-way RM ANOVA). These early post-CFC firing rate changes were not uniform across the population of CA1 neurons. While the majority of neurons recorded from CFC mice (>60%) showed firing rate increases across all states, 25–35% showed decreases, and a much smaller minority (<5% of neurons from all recordings) showed no change (less than 5% increase or decrease from baseline; **Figure 3B**). Post-CFC firing rate changes were maintained across the 24 h period following CFC (**Figure 3C**; main effect of conditioning $p < 0.05$, $p < 0.01$, and $p < 0.005$ for wake, REM, and SWS, respectively; conditioning × time-of-day interaction $p < 0.005$, $p < 0.001$, and $p < 0.005$ for wake, REM, and SWS, respectively, two-way RM ANOVA). In contrast, firing rates for Sham mice did not change significantly during REM or wakefulness after Sham conditioning (main effect of conditioning *N.S.*, two-way RM ANOVA), and showed a slight but significant decrease after Sham conditioning within SWS ($p < 0.05$, two-way RM ANOVA). When firing rate changes post-conditioning were expressed as a percent change from baseline, significant differences between Sham and CFC were evident, with mean increases of >50% present in all states immediately after conditioning (**Figure 3D**). The tendency for increased firing among CA1 neurons after CFC was present throughout the entire 24 h post-conditioning recording period. Thus the duration of firing rate changes we see *in vivo* (lasting at least 24 h following conditioning) is consistent with the reported timecourse of CA1 neurons' intrinsic excitability changes observed *in vitro* after classical conditioning in various rodent models (Moyer et al., 1996; McKay et al., 2009, 2013).

CA1 FIELD ACTIVITY IS ALTERED DURING CFM CONSOLIDATION

To determine how hippocampal network activity patterns are affected during CFM consolidation, raw spectral power for CA1 LFPs were compared in each behavioral state between corresponding 6 h time windows in the baseline and post-conditioning periods (**Figure 4A**). Comparisons of percent changes in LFP power across frequency bands showed clear differences between CFC and Sham mice. To characterize these differences, power spectral changes from baseline were summed across delta (0.5–4 Hz), theta (4–12 Hz), and gamma (25–50 Hz) frequency bands. In the first 6 h post-conditioning, CFC showed smaller increases in delta-activity than Sham mice during both wakefulness and SWS, and larger increases in REM (**Figures 4A–B**). At subsequent time points, delta-power showed larger increases in CFC mice than Sham mice—an effect that was present across all states (main effect of conditioning $p < 0.01$, $p < 0.001$, and *N.S.* for wake, REM, and SWS, respectively; conditioning × time-of-day interaction $p < 0.001$ for all states, two-way RM ANOVA). Theta-frequency activity showed even larger increases following CFC, which were present in REM and wakefulness (but not SWS) throughout the post-CFC recording period (main effect of conditioning $p < 0.005$, $p < 0.001$, and *N.S.* for wake, REM, and SWS, respectively; conditioning × time-of-day interaction $p < 0.005$ for REM, *N.S.* for wake and SWS, two-way RM ANOVA). Because the range of theta-frequencies reported here is broader than that published in some studies (which can be restricted to a range as narrow as 4–7 Hz), we also compared changes in the 4–7 Hz band, with nearly identical results (main effect of conditioning $p < 0.001$, $p < 0.001$, and *N.S.* for wake, REM, and SWS, respectively; conditioning × time-of-day interaction $p < 0.001$, $p < 0.05$, and *N.S.* for wake, REM, and SWS, respectively, two-way RM ANOVA). Slight increases in gamma were also present following CFC, but these changes were restricted to REM (main effect of conditioning $p < 0.001$; conditioning × time-of-day interaction $p < 0.001$, two-way RM ANOVA).

To test whether state-specific changes in activity were proportional to firing rate changes following CFC, we compared firing rate and LFP power changes across individual CA1 recording sites in the first 6 h post-conditioning (a time point when most of these changes are either maximal, or near-maximal). For all states and all frequency bands quantified, there was no correlation between changes in LFP power and changes in firing rate ($p > 0.1$ for all measures, Pearson correlation).

CA1 NETWORK STRUCTURE IS STABILIZED DURING CFM CONSOLIDATION

Recent quantitative analysis of hippocampal immediate-early gene expression has indicated that activity across the network of CA1 neurons during rest reflects the specific network activation patterns generated in prior waking experience (Marrone et al., 2008). This suggests that a memory trace, or engram, may be continuously present in CA1 at the level of network activation (and perhaps network functional connectivity) long after a learning experience ends. To test whether this is true of the CA1 network following CFC, we used a functional clustering algorithm (FCA; Feldt et al., 2009) to quantify functional connectivity between CA1 neurons. We also used minute-to-minute comparisons of CA1 network functional connectivity to quantify the stability of network activity patterns at baseline and after conditioning.

Stability of network functional structure was assessed over time by comparing FCA-generated network architecture between successive 1 min intervals across the entire baseline and post-conditioning periods (**Figure 5A**). The term "stability" in this case means the similarity (in time) of functional connectivity among recorded neurons. In this sense, more stable networks maintain similar functional clusters over time (i.e., the same neurons remain within given functional clusters, and neither the number of clusters nor the joining distance change significantly). Using this metric, average minute-to-minute stability values were calculated across each recording period, and changes in average stability across the recorded CA1 network post-conditioning were expressed as a percent change from baseline in each mouse. CA1 network structure stability increased significantly after conditioning in CFC mice, but not in Sham mice (**Figure 5B**). This stability increase was evident when all successive 1 min intervals were included in stability analysis, regardless of behavioral state, and also when only intervals spent in SWS were analyzed separately ($p < 0.01$ and $p < 0.005$ respectively for all-state and SWS-specific analysis, Student's *t*-test). However, the same stability increase was not seen across post-CFC intervals of wakefulness (stability changes for CFC vs. Sham *N.S.*, Student's *t*-test). Stability across

REM intervals could not be separately assessed due to the relatively brevity and infrequency of REM episodes, which reduced the reliability of stability measurements.

DISCUSSION

These studies were aimed at assessing sleep-associated changes in CA1 network activity that might contribute to the sleep-dependence of CFM consolidation. We found that single-trial CFC induces CFM without altering sleep architecture over the 24 h CFM consolidation period. CFC does, however, lead to three long-lasting changes in either the activity of individual CA1 neurons or the interactions of these neurons within the hippocampal network. These changes are discussed in detail below:

POST-CFC FIRING RATE INCREASES IN CA1 NEURONS

First, we find that neuronal firing rates increase immediately after CFC *in vivo*, and remain elevated over the course of 24 h of post-CFC recording. This increase was specific to CFC, as similar firing rate changes were not seen after Sham conditioning, where exploration of a novel context is not paired with foot shock. Critically, the time course of these changes is similar to that of excitability changes measured *in vitro* in mouse, rat, and rabbit CA1 neurons following aversive conditioning (Moyer et al., 1996; McKay et al., 2009, 2013).

Recent studies of the underlying mechanisms for these changes have shown that aversive conditioning leads to decreased expression of KCNN2, an apamin-sensitive SK channel, in the hippocampus (McKay et al., 2012). Activation of SK channels blocks conditioning-induced excitability changes in both CA1 pyramidal neurons and interneurons, and impairs learning (McKay et al., 2012, 2013). Because SK channels play a critical role in regulating after hyperpolarizing (AHP) currents and thus firing rates in CA1 (Pedarzani et al., 2005), excitability changes associated with SK channel reductions may mediate the firing rate increases we see *in vivo* after CFC. Critically, sleep may play an important role in maintaining conditioning-induced reductions in SK channel expression. A recent study examining sleep- and sleep deprivation-mediated gene expression changes found that expression of KCNN2 is reduced during sleep and increased during sleep deprivation in multiple brain areas (Mackiewicz et al., 2007). Such a mechanism may explain the effect of sleep loss in reducing membrane excitability in CA1 (McDermott et al., 2003).

What effect do firing rate increases have on the hippocampal network during CFM consolidation? While CA1 network activity in the hours following CFC is essential for CFM (Daumas et al., 2005), the role of increased activity in the consolidation process is unknown. One possibility is that increased firing rates among CA1 neurons drives synaptic plasticity in the network throughout the post-CFC consolidation window. Firing rate contributes to the sign (LTP or LTD) of spike-timing-dependent plasticity (Feldman, 2012), and for some CA1 synapses, firing rate appears to be more important than pre-vs.-postsynaptic spike timing for determining the sign of plasticity (Wittenberg and Wang, 2006). Based on available data from *in vitro* studies, it seems plausible that the increase in firing we observe after CFC *in vivo* biases CA1 neurons toward synaptic potentiation, which appears to be essential for multiple forms of CA1-dependent memory. Interference with cellular pathways required for synaptic potentiation in the hours following CFC (through either drug treatments, or behaviorally through sleep deprivation) impairs CFM consolidation (Bourtchouladze et al., 1998; Vecsey et al., 2009), and postsynaptic potentiation has been measured within CA1 *in vivo* within 6 h following object recognition training (Clarke et al., 2010). Because synaptic potentiation clearly plays an important role in long-term memory formation, it stands to reason that enhanced neuronal firing could promote consolidation through this mechanism.

POST-CFC INCREASES IN NETWORK OSCILLATIONS

Second, we find that LFP oscillations in CA1 are specifically increased in multiple frequency bands following CFC (relative to Sham conditioning), with a time course similar to that seen for firing rate changes (i.e., lasting up to 24 h). While relatively modest changes were seen for gamma-oscillations, which were significantly increased following CFC only during REM sleep. In terms of magnitude, the largest changes in LFP activity were increases in the theta (4–12 Hz) range, particularly during REM sleep. CA1 neurons show natural oscillations in membrane potential *in vitro* that resonate with exogenous input at theta-frequencies (Leung and Yu, 1998), and CA1 theta-oscillations *in vivo* are specifically augmented during novel experiences which engage the hippocampus (Penley et al., 2013). Because theta-frequency stimulation can induce LTP in CA1 *in vitro* (Woo et al., 2000), and driving theta-oscillations in the hippocampus *in vivo* promotes memory consolidation (Wetzel et al., 1977), it is tempting to speculate that these oscillations drive the network plasticity underlying memory formation. Our data suggest that naturally-occurring enhancements in theta-frequency CA1 oscillations accompany CFM consolidation. These increases are most prominent following CFC in REM sleep (a state in which prominent hippocampal theta-oscillations are a consistent feature), but are also clearly present in wakefulness. These findings raise two questions. First, what network changes underlie these long-term increases in theta-frequency activity? One possibility is that CFC leads to rapid alterations in hippocampal inputs (e.g., input from the medial septum) which could subsequently drive theta-activity more robustly in CA1 (Hasselmo, 2005). Another is that CFC alters intra-hippocampal network connectivity, which could lead to more coherent theta-oscillatory activity. While our current data do not address the former, our analyses of CA1 network structure (see below) suggest that the latter possibility is plausible. A second question is whether, and how, these oscillations contribute to memory consolidation. As is true for increases in neuronal firing, it seems plausible that enhancing theta-oscillations could promote consolidation by creating optimal conditions for synaptic potentiation within hippocampal circuits. Moreover, because increased theta-frequency coherence between the hippocampus and other brain areas (e.g., the amygdala and prefrontal cortex) is specifically associated with other forms of memory consolidation (Benchenane et al., 2010; Popa et al., 2010), it is also possible that enhanced CA1 theta-rhythms could drive systems-level memory consolidation during sleep (Aton et al., 2009b).

POST-CFC STABILIZATION OF THE CA1 NETWORK

The third change we observe in CA1 after CFC is a significant stabilization of functional connectivity within the CA1 network. Network stability increases after conditioning were not seen in any of the Sham mice, but were present in all CFC mice. Importantly, and in contrast to the firing rate and LFP changes we see following CFC, significant increases in network stability after CFC were *not* seen during periods of wakefulness, but were clearly seen in SWS. Thus increased network stability is one feature of network activity that is associated specifically with sleep (and not associated with wakefulness) during CFM consolidation. Because sleep is necessary for long-term memory formation in this system, it stands to reason that network-level changes associated with sleep are critical for the consolidation process. Stabilization of spike-timing relationships within CA1 may be a plausible strategy for preserving a memory trace (i.e., an engram) of CFC during consolidation. In this case, the memory trace would be weakened by replacing sleep with wakefulness (i.e., with sleep deprivation)—which could provide a network-level mechanistic explanation for why sleep deprivation disrupts mnemonic function.

"Replay" of experience-associated sequences of network activity during subsequent rest has been hypothesized to play an important role in memory consolidation (Abel et al., 2013). However, most studies of replay involve recording activity patterns from animals following repetition of a relatively familiar task; i.e., the animals under study have been trained over a period of days to weeks prior to recording. Thus the replay described in these studies is not temporally associated with consolidation of new memories; it might more fairly be described as occurring following practice of a very familiar task. In almost no case has sequential pattern reactivation been demonstrated in the context of active consolidation of memory following a novel learning experience. While the stability measure reported here does not necessarily require *sequential* reactivation of specific neuronal firing patterns, it does quantify the degree to which the pattern of functional connectivity (based on relative spike timing) among recorded neurons remains stable over time. Critically, the increase in stability we see following CFC is maintained across the entire 24 h post-CFC period, while sequential replay is typically reported for only a few minutes following experience (Aton et al., 2009b). Thus the longer-term stabilization of network activity patterns we see following CFC may be a true network-level correlate of *de novo* memory consolidation. The fact that increased stability is associated specifically with a behavioral state required for consolidation suggests that it may play a functional role in protecting memory traces at their most labile state, across the first 24 h following encoding.

Taken together, the neuronal and network activity changes we have found comprise one of the first descriptions of *in vivo* electrophysiological changes in CA1 corresponding to active fear memory consolidation. Future studies will be aimed at better understanding whether similar network level changes occur following learning in other brain structures, the role of sleep in promoting these changes, as well as which network-level changes are necessary, and sufficient, for memory consolidation.

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BMAL1 controls the diurnal rhythm and set point for electrical seizure threshold in mice

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The epilepsies are a heterogeneous group of neurological diseases defined by the occurrence of unprovoked seizures which, in many cases, are correlated with diurnal rhythms. In order to gain insight into the biological mechanisms controlling this phenomenon, we characterized time-of-day effects on electrical seizure threshold in mice. Male C57BL/6J wild-type mice were maintained on a 14/10 h light/dark cycle, from birth until 6 weeks of age for seizure testing. Seizure thresholds were measured using a step-wise paradigm involving a single daily electrical stimulus. Results showed that the current required to elicit both generalized and maximal seizures was significantly higher in mice tested during the dark phase of the diurnal cycle compared to mice tested during the light phase. This rhythm was absent in BMAL1 knockout (KO) mice. BMAL1 KO also exhibited significantly reduced seizure thresholds at all times tested, compared to C57BL/6J mice. Results document a significant influence of time-of-day on electrical seizure threshold in mice and suggest that this effect is under the control of genes that are known to regulate circadian behaviors. Furthermore, low seizure thresholds in BMAL1 KO mice suggest that BMAL1 itself is directly involved in controlling neuronal excitability.

Keywords: ARNTL transcription factors, circadian rhythm, CLOCK proteins, epilepsy, epileptogenesis, MOP3

INTRODUCTION

Time-of-day factors such as sleep and circadian rhythms are well known to influence expression of seizures (Matos et al., 2010a; Cho, 2012). Studies in humans have shown an association between seizures and alterations in sleep-wake states (Bazil and Walczak, 1997; Crespel et al., 2000; Herman et al., 2001; Dinner, 2002). In patients with common forms of idiopathic generalized epilepsy, tonic, and tonic-clonic seizures are more frequently seen in sleep, whereas all other generalized semiologic seizure types including clonic, myoclonic, absence and atonic occur more frequently out of wakefulness (Zarowski et al., 2011), suggesting circadian rhythms control distinct mechanisms of neuronal hyperexcitability that may result in seizures. Sleep is also associated with twice as many interictal discharges in patients with idiopathic generalized epilepsy, especially during the period of nonrapid eye movement sleep (Seneviratne et al., 2012). In patients with lesional epilepsy characterized by seizures originating from the frontal lobe, nearly three quarters of their seizures occur during sleep (Kaleyias et al., 2011). Furthermore, sleep not only increases the frequency of epileptiform abnormalities, but also alters electrographic seizure morphology and distribution (Sanchez Fernandez et al., 2013). Seizures can also influence changes in sleep. Epileptic patients demonstrate multiple sleep abnormalities, including increased sleep latency, fragmented sleep, increased awakenings and stage shifts, and an increase in stages 1 and 2 of nonrapid eye movement sleep (Foldvary-Schaefer and Grigg-Damberger, 2009).

Among many forms of human epilepsy, several associations between seizure expression and time-of-day are especially well documented. Included are benign Rolandic epilepsy of childhood, in which seizures occur most prominently during sleep (Beaussart, 1972), Autosomal Dominant Nocturnal Frontal Lobe Epilepsy (ADNFLE) in which seizures are most prominent following transition from wakefulness to sleep (Scheffer et al., 1994, 1995) and juvenile myoclonic epilepsy when seizures occur mostly in the transition from sleep to wakefulness (Gigli et al., 1992). In patients with non-lesional temporal lobe epilepsy (TLE), the highest frequency of seizures was observed to occur approximately in the middle of the wake period, peaking at 1500 h (Quigg et al., 1998). This is consistent with results from two other studies, one in which patients with TLE exhibited a peak in seizure frequency in the afternoon between 1500–1900 h (Pavlova et al., 2004) and another in patients with focal epilepsies experienced significantly greater numbers of seizures between 1100–1700 h (Hofstra et al., 2011). Evidence for bimodal increase in seizure frequency in TLE has also been reported with peaks in the morning and in the late afternoon (Durazzo et al., 2008; Karafin et al., 2010). Therefore, understanding the way sleep or circadian

mechanisms play a role in time-of-day regulation of seizure occurrence has important clinical applications.

Associations between sleep and epilepsy are also observed in animal models (van Luijtelaar and Bikbaev, 2007; Matos et al., 2010a,b; Yi et al., 2012). Mice with targeted deletion of genes involved in diurnal rhythms have been particularly instructive. Spontaneous electrographic seizures were documented in mice lacking Pten, a tumor suppressor gene that also significantly affects free-running rhythm (Ogawa et al., 2007). Deletion of three distinct PAR bZip transcription factors, proteins that accumulate with strong diurnal rhythms in critical brain areas such as the suprachiasmatic nucleus of the hypothalamus, causes spontaneous seizures in mice and is associated with reduced threshold to audiogenic seizures (Gachon et al., 2004). Interestingly, interictal abnormalities, and occurrence of spontaneous behavioral seizures in triple knockout PAR bZip mice followed a circadian trend that paralleled the distribution of sleep. These data suggest that molecular mechanisms related to the circadian clock likely play a key role in establishment of seizure susceptibility.

BMAL1 (also known as Arntl or Mop3) is a basic helix-loop-helix-PAS domain containing transcription factor and a core component of the circadian clock (Ko and Takahashi, 2006). BMAL1 gene expression is necessary for the establishment of circadian rhythms under free-running conditions (Bunger et al., 2000). BMAL1 KO mice have a significant disruption of the normal diurnal distribution of NREM and REM sleep over the light-dark cycle (Laposky et al., 2005), suggesting that BMAL1 is necessary for time-of-day dependent behavior even in the presence of a zeitgeber. BMAL1 expression has also been shown to be affected by sleep loss (Maret et al., 2007), and can influence the homeostatic response to sleep deprivation (Laposky et al., 2005) and certain aspects of memory formation (Kondratova et al., 2010). In addition to sleep and memory, core molecular clock mechanisms influence synaptic plasticity, further evidence for a neurophysiological role outside of the establishment of circadian locomotor rhythms (Gerstner and Yin, 2010). In this study, we hypothesized that seizure threshold has a time-of-day dependent component regulated by BMAL1. We observed a strong diurnal regulation of both generalized and maximal seizure threshold in WT mice. This effect was blocked in the BMAL1 KO. We also observed that seizure endpoints are largely influenced by the light-dark cycle, and that in addition to the control of diurnal seizure susceptibility, BMAL1 is also necessary for baseline seizure threshold. Further, BMAL1 KO mice display reduced seizure endpoints, suggesting clock factors such as BMAL1 affect not only the temporal patterning of seizures, but also overall neural excitability.

METHODS

ANIMALS

All studies were approved by the Institutional Animal Care and Use Committees at the University of Pennsylvania and VAMC Coatesville. Experiments involved C57BL/6J wild type mice (The Jackson Laboratory, Bar Harbor, ME) and co-isogenic Bmal knockout (KO) mice (from G. FitzGerald). Mice used in these studies were bred in-house at the VAMC Coatesville vivarium. Bmal KO mice were maintained as a homozygous strain. Litters were weaned between 21–28 days and pups were group housed by sex until the age of 6 weeks when they were entered into the study. Mice were maintained on a 14/10 h light dark cycle with free access to food and water at all times.

ZEITGEBER ENTRAINMENT

At the age of 6 weeks, mice were randomly assigned into one of 6 experimental groups ($N = 6$ mice per group). Mice were entrained to 14:10 h light:dark cycles for 2 weeks prior to seizure testing. Zeitgeber time (ZT) 1 is defined as 1 h following lights on in the 14:10 light:dark cycle. Bmal KO mice were tested under two conditions, ZT1 and ZT15 ($N = 7$ and 6 per group, respectively).

SEIZURE TESTS

Seizure testing was initiated when mice reached the age of 8 weeks and due to the variable effect of estrous cycle on seizure susceptibility, only male mice were studied. The electrical thresholds for generalized and maximal seizures were measured as described previously (Ferraro et al., 2011). Mice were tested with a single electric shock delivered via ear clip electrodes once per day. Sham mice received the same handling, but did not receive the shock. We used a constant current electroshock unit (model No. 7801, Ugo Basile, Varese, Italy) in which the initial current level was set at 20 mA and increased by 2 mA with each successive daily trial until a maximal seizure was observed. Other parameters of the stimulus were held constant (60 Hz, 0.4 ms pulse width, 0.2 s duration). Seizures were elicited at all current intensities utilized; lower intensities produced facial and forelimb clonus, whereas higher intensities produced generalized and maximal seizures. A generalized seizure was defined by loss of upright posture (i.e., falling over) and bilateral limb clonus. The current value at which mice first exhibited a generalized seizure was taken as the generalized electroshock seizure threshold (GEST). A maximal seizure was defined by bilateral tonic extension of hind limbs. The current value at which mice exhibited tonic hind limb extension was taken as the MEST. The sequence of responses that characterized a trial in which a maximal seizure was observed is as follows: bilateral tonic forelimb flexion, bilateral tonic hind limb flexion and bilateral tonic hind limb extension. These signs were sometimes preceded or accompanied by wild running in the observation chamber. Mice were euthanized by cervical dislocation under CO2 anesthesia immediately after a trial in which a maximal seizure was elicited.

WESTERN BLOTTING

For tissue punches of hippocampus, hemisectioned brains were embedded rostral side down in OCT media and sectioned on a Leica (CM3050) cryostat at −25°C, and a 500–1000 μm 1 mm diameter punch from Interaural 1.10 to 2.10 mm (according to Paxinos & Franklin Mouse Brain Atlas, 2nd ed.) was taken for each MEST and Sham mouse from ZT1 and ZT15. Individual hippocampal punches were homogenized in 100 μl Tris-HCl buffer with Protease Inhibitor Cocktail (Sigma P2714) for 45 s and centrifuged at 14,000 rpm for 1 min, and 5 μl lysates were loaded with 5 μl 2X loading buffer and separated on 10% Tric-HCl 0.75 mm gels and transferred to nitrocellulose membranes (Invitrogen). The membranes were blocked with 5% nonfat

dry milk (Nestlé) and blotted with BMAL (Arntl) antibody (1:1000; Bethyl Laboratories) and beta-actin antibody (1:1000; Cell Signaling). Goat anti-Rabbit IR800 (1:5000; LI-COR) secondary antibody was used to visualize on Odyssey scanner (LI-COR). Intensities of protein were determined using ImageJ software (NIH).

DATA ANALYSIS

Thresholds for generalized and maximal seizures were determined as arithmetic mean values for each experimental group. ANOVA was used to examine the effect of time of day on seizure thresholds and a 2-Way ANOVA was used to include the effect of strain. *Post hoc* analyses to examine statistical relationships for seizure threshold values between individual groups were conducted using the Tukey test. For analysis of Bmal KO data, comparisons were made to values from B6 mice collapsed across lights-on and lights-off phases since there were no significant differences between groups within each phase.

Kaplan-Meier analysis for seizure endpoints was examined using MedCalc statistical software (http://www.medcalc.org), and trial time was defined as the day animals reached either generalized or maximal seizure endpoint.

For diurnal western blot studies statistical significance was determined using a two-tailed Student's *t*-test using Excel (Microsoft).

RESULTS

Seizure threshold was examined at 6 timepoints across a 14:10 light:dark cycle for diurnal fluctuations in wild-type (WT) mice. We observed a significant time-of-day dependent change in seizure threshold in both generalized (GEST) and maximal (MEST) seizures ($p = 0.023$ and $p = 0.004$, respectively, One-Way ANOVA), with elevated threshold levels in the dark phase (**Figures 1A,B**). Peak GEST and MEST each occurred 1 h into the dark phase at ZT15 ($p < 0.05$, Student-Newman-Keuls *post-hoc* test). Since GEST and MEST values appeared lower during the light phase compared to the dark phase overall, we were interested in testing for the diurnal effects on seizure endpoints between these two groups. We observed a significant decrease in no response probability for both GEST and MEST during the light phase compared to the dark phase (**Figures 2A,B**; $p = 0.0008$ and $p = 0.0002$, respectively, Kaplan-Meier analysis Logrank test).

Circadian clock genes, such as BMAL1, are known to regulate circadian rhythms of locomotor wheel-running behavior (Bunger et al., 2000), as well as the diurnal control of sleep-wake states (Laposky et al., 2005) and memory formation (Kondratova et al., 2010), suggesting that these genes may also participate in basic neurophysiological function. Therefore we examined the effects of diurnal seizure susceptibility in BMAL1 KO mice, and found that the time-of-day variation in both GEST and MEST observed in WT mice was blocked (**Figures 3A,B**). Further, the baseline setpoint for seizure threshold was also significantly reduced in BMAL1 KO mice compared to WT mice for both GEST and MEST (**Figures 3A,B**). BMAL1 KO mice had a significant reduction in GEST irrespective of time-of-day (**Figure 3A**, $p < 0.05$ ZT1; $p < 0.01$ ZT15, two-tailed *t*-test), while MEST was significantly lower at ZT15 compared to WT (**Figure 3B**, $p < 0.001$ two-tailed *t*-test).

We next wanted to determine whether there was a difference in seizure end-point probability in BMAL1 KO mice. Given that the

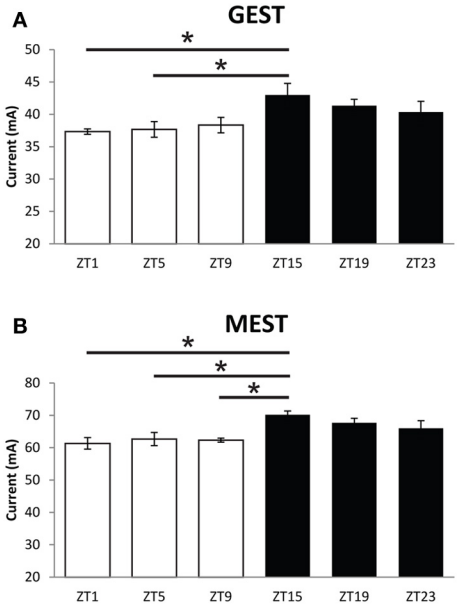

FIGURE 1 | Diurnal effects on seizure threshold. Time-of-day variation in **(A)** generalized (GEST) and **(B)** maximal (MEST) electroshock seizure threshold. Male C57BL/6J mice (8–9 weeks of age) were housed individually and tested for electroshock seizure thresholds at different times during the 14:10 Lights-on:Lights-off diurnal cycle. One-Way ANOVA for GEST, $p = 0.023$, MEST, $p = 0.004$; ($N = 6$). Student-Newman-Keuls *post-hoc* test for all pairwise comparisons are shown for differences between groups for GEST and MEST (*$p < 0.05$). ZT, zeitgeber time. Light bars = Lights on, Dark bars = Lights off.

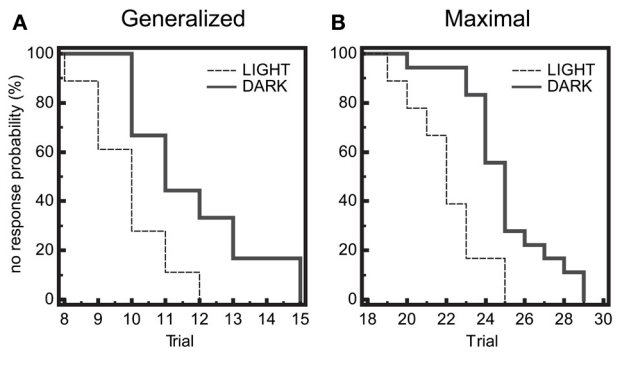

FIGURE 2 | Diurnal effects on seizure threshold. Endpoints of electroshock-induced seizures for the three light-period groups (ZT1, 5, 9) and dark-period groups (ZT15, 19, 23) show differential percent probability of no response in both **(A)** generalized (GEST) and **(B)** maximal (MEST) electroshock seizure threshold, with greater resistance in the dark-phase (solid line) compared to the light phase (dashed line). **(A)** Median trial number to reach GEST: Light = 10, Dark = 11; Logrank test: Chi-square 11.31, $P = 0.0008$; $N = 18$ per group. **(B)** Median trial number to reach MEST: Light = 22, Dark = 25; Logrank test: Chi-square 13.96, $P = 0.0002$; $N = 18$ per group.

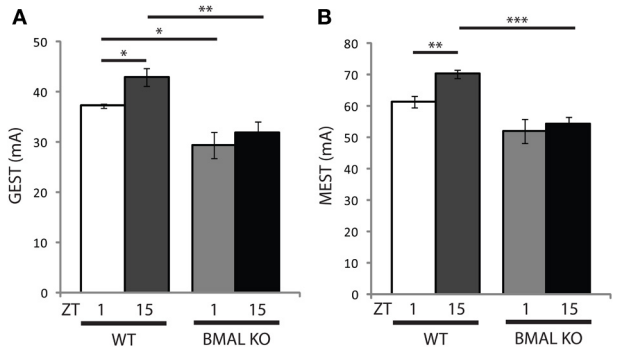

FIGURE 3 | Diurnal seizure susceptibility and baseline setpoint depends on BMAL1 expression. Time-of-day variation in **(A)** generalized (GEST) and **(B)** maximal (MEST) electroshock seizure threshold observed in wild-type (WT) mice is blocked in BMAL KO mice. **(A)** *t*-test (two-tail) WT GEST: ZT1 vs. ZT15 $^{*}p < 0.05$; WT vs. BMAL KO GEST: ZT1 vs. ZT1 $^{*}p < 0.05$, ZT15 vs. ZT15 $^{**}p < 0.01$. **(B)** *t*-test (two-tail) WT MEST: ZT1 vs. ZT15 $^{**}p < 0.01$; WT vs. BMAL KO GEST: ZT15 vs. ZT15 $^{***}p < 0.001$. Error bars = s.e.m. ZT, zeitgeber time.

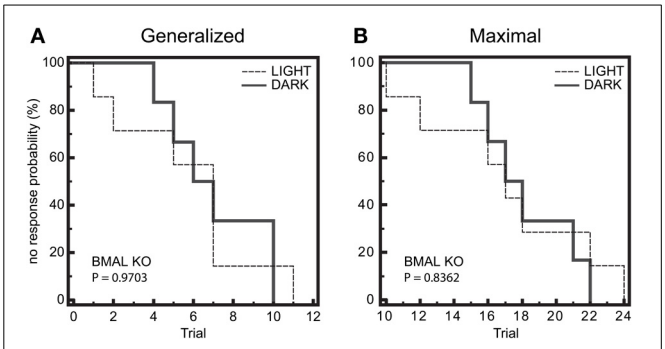

FIGURE 4 | Diurnal effects on seizure endpoints are blocked in BMAL1 KO mice. (A) and **(B)** Endpoints of electroshock-induced seizures for the BMAL KO mice in the light-period (ZT1) and dark-period (ZT15) fail to show differential percent probability of no response in either **(A)** generalized (GEST) or **(B)** maximal (MEST) electroshock seizure threshold. **(A)** Median trial number to reach GEST: Light = 7, Dark = 7; Logrank test, $P = 0.9703$; $N = 6$–7 per group. **(B)** Median trial number to reach MEST: Light = 17, Dark = 18; Logrank test, $P = 0.8362$; $N = 6$–7 per group.

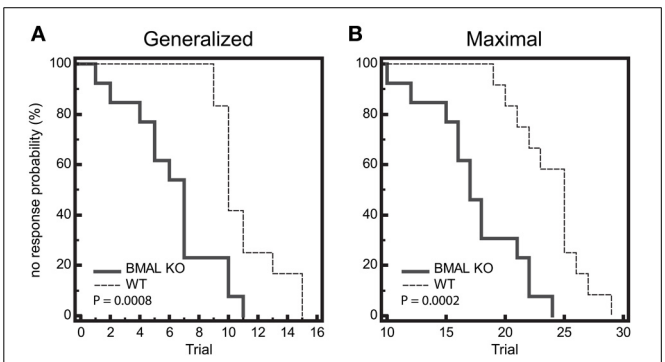

FIGURE 5 | Seizure endpoints are reduced in BMAL1 KO mice compared to WT. (A) and **(B)** Endpoints of electroshock-induced seizures comparing BMAL KO mice and WT mice from data combined in for the light-period and dark-period (ZT1 and ZT15) show differential percent probability of no response in both **(A)** generalized (GEST) and **(B)** maximal (MEST) electroshock seizure threshold. **(A)** Median trial number to reach GEST: WT = 10, BMAL KO = 7; Logrank test, $P = 0.0008$; $N = 12$–13 per group. **(B)** Median trial number to reach MEST: WT = 25, BMAL KO = 18; Logrank test, $P = 0.0002$; $N = 12$–13 per group.

diurnal profile of seizure threshold is blocked in BMAL1 KO mice, we hypothesized that the diurnal variation in no response probability should also be blocked. Kaplan-Meier analysis of seizure endpoints revealed that BMAL1 KO mice do not display a difference in seizure endpoint between light and dark phase groups for either GEST or MEST (**Figure 4**, $p = 0.9703$ and $p = 0.8362$, respectively, Logrank test). Since BMAL1 KO mice have a reduced seizure setpoint, we hypothesized that BMAL1 KO mice should also have reduced overall seizure endpoint values compared to WT mice. Kaplan-Meier analysis of seizure endpoints comparing BMAL1 KO mice with WT mice show a significant difference in seizure endpoint for both GEST and MEST (**Figure 5**, $p = 0.0008$ and $p = 0.0002$, respectively, Logrank test). These strain differences on GEST and MEST were also time-of-day dependent, since seizure endpoints at ZT1 did not significantly differ between BMAL1 KO mice and WT mice, but were significant at ZT15 (**Figure 6**, $p = 0.0023$ and $p = 0.0005$, respectively, Kaplan-Meier analysis Logrank test).

Lastly, we wanted to examine whether seizure activity in turn affects time-of-day dependent BMAL1 expression in WT mice. Brains of WT mice subjected to MEST or handled controls (Sham) from ZT1 and ZT15 were dissected, and punches of hippocampi from hemisections were lysed for analysis of protein expression by western blotting. The time-of-day variation in hippocampal BMAL1 protein expression in Sham controls (**Figure 7**, $p < 0.05$, *t*-test) also remained following MEST (**Figure 7**, $p < 0.01$, *t*-test), indicating seizure activity does not influence diurnal BMAL1 expression.

DISCUSSION

In this study, we observed a time-of-day dependent effect on seizure threshold for both generalized and maximal seizures, with a peak in threshold at the beginning of the dark cycle. We also observed a difference in seizure endpoints between the light period and dark period, suggesting circadian factors may contribute to neural excitability. Therefore, we next examined the time-of-day effect on seizure threshold in the circadian clock mutant BMAL1 KO mice, and observed the diurnal profile of GEST and MEST was blocked. Further, we show overall seizure threshold is significantly lower in BMAL1 KO mice compared to WT mice, suggesting BMAL1 regulates baseline levels of excitability in addition to time-of-day dependent changes. Seizure endpoints for both GEST and MEST were significantly reduced in BMAL1 KO mice compared to WT mice. The diurnal hippocampal expression of BMAL1 protein was not affected by seizure activity. These findings suggest time-of-day regulates seizure susceptibility, and this effect is mediated, at least in part, by core circadian factors such as BMAL1. To the best of our knowledge, this is the first report to characterize an association between a core clock gene product and epileptogenesis.

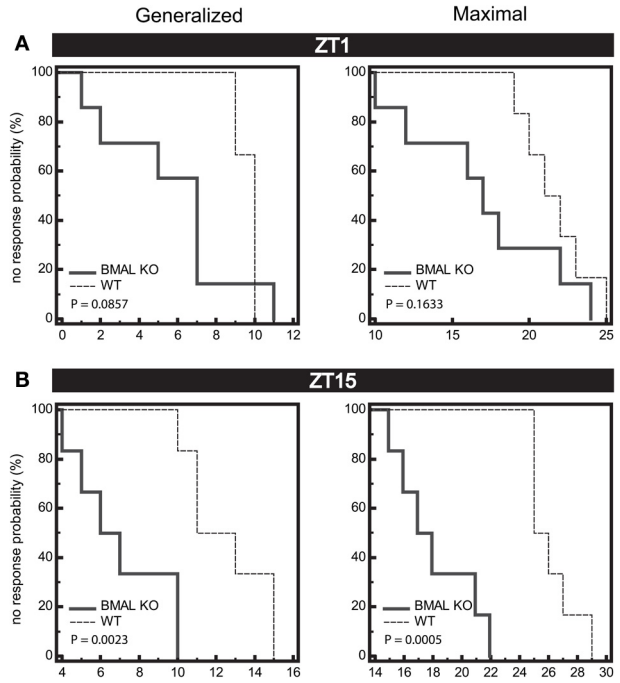

FIGURE 6 | Seizure endpoints are reduced in BMAL1 KO mice compared to WT. (A) and **(B)** Endpoints of electroshock-induced seizures comparing BMAL KO mice and WT mice from data combined in for the light-period (**A**, ZT1) and dark-period (**B**, ZT15) show differential percent probability of no response in both generalized (GEST) and maximal (MEST) electroshock seizure threshold. **(A)** ZT1, Median trial number to reach GEST: WT = 10, BMAL KO = 7; Logrank test, $P = 0.0857$; Median trial number to reach MEST: WT = 22, BMAL KO = 17; Logrank test, $P = 0.1633$, $N = 6$ per group. **(B)** ZT15, Median trial number to reach GEST: WT = 13, BMAL KO = 7; Logrank test, $P = 0.0023$; Median trial number to reach MEST: WT = 26, BMAL KO = 18; Logrank test, $P = 0.0005$; $N = 6$–7 per group.

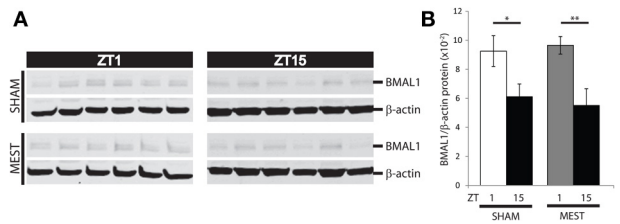

FIGURE 7 | Diurnal hippocampal BMAL1 expression is not affected by MEST. Time-of-day variation in BMAL1 hippocampal protein expression is unaffected by MEST. **(A)** Western blot of WT mouse hippocampal BMAL1 and b-actin in SHAM controls vs. MEST at ZT1 and ZT15. **(B)** Quantification of BMAL/b-actin protein expression from western blots in **(A)**; *t*-test (two-tail) SHAM: ZT1 vs. ZT15 $*p < 0.05$; MEST: ZT1 vs. ZT15 $**p < 0.01$. $N = 6$ per group. Error bars = s.e.m. ZT, zeitgeber time.

Sleep-wake behavior and circadian rhythms are known to regulate propensity of seizures, while their relative influence likely depends on origin and seizure type (Matos et al., 2010a; Cho, 2012). For example, adults with complex partial and temporal seizures were observed to occur more frequently during the 11:00–17:00 h time period, while parietal seizures were more prevalent during the 17:00–23:00 period (Hofstra et al., 2009a). Age-specific and time-of-day dependent differences in extra-temporal seizures were observed between children and adults (Hofstra et al., 2009b), suggesting developmental regulation of the diurnal susceptibility for certain seizures. Generalized seizures in children were observed more often during sleep for tonic or tonic-clonic types, whereas absence, atonic, myoclonic, and clonic seizures occurred more readily during wakefulness (Zarowski et al., 2011). This evidence indicates that seizure type, severity, and developmental profile are all additional factors which may differentially contribute to the time-of-day variation in seizure susceptibility. Future work using animal models to examine molecular mechanisms that differentially regulate diurnal profiles between seizure subtypes may help determine better treatments tailored to individuals.

Previous studies examining the deletion of three genes that are regulated by the circadian clock show increased susceptibility to generalized spontaneous seizures and audiogenic epilepsies (Gachon et al., 2004), suggesting clock output can modulate time-of-day expression of seizures. Animal models of epilepsy also exhibit circadian rhythms in seizures (Hellier and Dudek, 1999; Fenoglio-Simeone et al., 2009; Tchekalarova et al., 2010; Matzen et al., 2012). KCNA1 KO mice show robust oscillations in diurnal seizures, with heightened levels near the dark-light transition, and a lower propensity at the light-dark transition (Fenoglio-Simeone et al., 2009), a time-of-day effect corresponding to our observation of elevations in WT mice seizure threshold in this study. Here, we observed that this diurnal influence of seizure threshold was absent, and the overall threshold was further reduced in BMAL1 KO mice. BMAL1 KO mice have disrupted rest-activity cycles under light-dark conditions (Laposky et al., 2005). Since KCNA1 KO mice also have disrupted rest-activity cycles (Fenoglio-Simeone et al., 2009), but unlike BMAL1 KO mice, have robust diurnal rhythms of seizures, these studies may point toward potential mechanisms dissociating circadian effects from sleep/wake effects. Our work demonstrates that overall seizure susceptibility is regulated by time-of-day, and this effect is mediated by a core clock gene. Future studies examining development of the diurnal profile, and relative impact of factors that specifically affect either sleep or the circadian clock will be important to disentangle the principal effect of BMAL1 on seizure threshold.

Animal models also show a reciprocal interaction between sleep and epilepsy (van Luijtelaar and Bikbaev, 2007; Matos et al., 2010a,b; Yi et al., 2012), evidence for the importance of the utility of animal models in translational research studies in this area (Matos et al., 2011). Some studies on models of limbic epilepsy have paralleled human studies in that spontaneously occurring seizures are more frequent during the light phase of the diurnal cycle. For example, pilocarpine-treated rats exhibited twice as many spontaneous seizures during the light phase as compared to the dark phase, a rhythm that remained intact and governed by the clock even when animals were maintained in constant darkness (Quigg, 2000). However, a more recent study reported spontaneous seizures elicited by pilocarpine treatment in rats was independent of the circadian cycle (Bajorat et al., 2011), an effect likely due to dependence on underlying sleep architecture (Matos et al., 2010b). In a model of electrically-induced status

epilepticus, dentate gyrus neurons exhibited enhanced electrical excitability that corresponded with the time of day of enhanced seizure susceptibility (Matzen et al., 2012). Further, in a study of WAG/Rij spontaneously epileptic rats, an 8-h shift in onset of the light phase resulted in prolonged aggravation of epileptic activity, observed mostly during the light phase (Smyk et al., 2012). In a study that observed a time-of-day distribution of spontaneous motor seizures in rats, a difference in the relative number of seizures when rats were "active" vs. "inactive" also occurred independent of time-of-day (Hellier and Dudek, 1999). This indicates there may be distinct sleep/wake state behavioral components separate from circadian influence on seizure susceptibility. These studies also raise the importance of using animal models for examining the relationship between time-of-day behaviors and predisposition to seizure.

Diurnal factors, such as sleep and circadian behavior are known to impact the propensity of seizures. Many studies have reported seizure activity is increased by sleep, or is more pronounced after arousal from sleep or following sleep deprivation. Time-of-day dependent differences in seizure could result from distinct mechanisms related to either sleep or circadian factors. In addition, whether an association between core circadian clock factors and epilepsy exists is unknown. Here, we observed that seizure susceptibility and endpoints are driven strongly by time-of-day dependent factors. As a first step in elucidating potential mechanisms responsible for these observations, we identified the core-clock transcription factor BMAL1 gene to be necessary for these time-of-day differences in seizure threshold and endpoint. Further, while seizure activity itself does not influence hippocampal BMAL1 expression, we show the BMAL1 gene regulates baseline threshold for both generalized and maximal seizures. These studies implicate circadian-clock signaling pathways in mediating seizure susceptibility and neural excitability, providing needed insight into molecular mechanisms contributing to epileptogenesis (Di Maio, 2014) and a new model organism from which to study the relationship between time-of-day dependent behaviors and seizure.

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Circadian gating of neuronal functionality: a basis for iterative metaplasticity[1]

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Brain plasticity, the ability of the nervous system to encode experience, is a modulatory process leading to long-lasting structural and functional changes. Salient experiences induce plastic changes in neurons of the hippocampus, the basis of memory formation and recall. In the suprachiasmatic nucleus (SCN), the central circadian (~24-h) clock, experience with light at night induces changes in neuronal state, leading to *circadian plasticity*. The SCN's endogenous ~24-h time-generator comprises a dynamic series of functional states, which gate plastic responses. This restricts light-induced alteration in SCN state-dynamics and outputs to the nighttime. Endogenously generated circadian oscillators coordinate the cyclic states of excitability and intracellular signaling molecules that prime SCN receptivity to plasticity signals, generating nightly windows of susceptibility. We propose that this constitutes a paradigm of ~24-h *iterative metaplasticity*, the repeated, patterned occurrence of susceptibility to induction of neuronal plasticity. We detail effectors permissive for the cyclic susceptibility to plasticity. We consider similarities of intracellular and membrane mechanisms underlying plasticity in SCN circadian plasticity and in hippocampal long-term potentiation (LTP). The emerging prominence of the hippocampal circadian clock points to iterative metaplasticity in that tissue as well. Exploring these links holds great promise for understanding circadian shaping of synaptic plasticity, learning, and memory.

Keywords: circadian rhythms, gating, plasticity, iterative metaplasticity, hippocampus, suprachiasmatic nucleus, glutamatergic, signaling

INTRODUCTION

The ability of salient stimuli to induce persistent changes in the structure and function of neurons is a fundamental modulatory process that confers the ability to modify physiology and behavior, learning from experience (Markham and Greenough, 2004). Although observed throughout the brain, plasticity is best studied in the hippocampus, a site critical to establishing and recalling new memories. Cellular and molecular mechanisms by which stimuli generate synaptic changes in hippocampal sub-regions fall into two classes: those that cause long-term potentiation (LTP) vs. long-term depression (LTD). Plastic changes in the hippocampus develop along linear timelines, initiated by the stimulus, followed by multiple sequential steps that are necessary to establish long-term, persistent functional changes.

Among the most salient stimuli for life on Earth are the alternating environmental states of day and night. The cycle of day and night partitions the availability of energy, both thermal and nutrient. Alternating activity vs. rest behaviors align with these environmental states. Internally, day-night changes in metabolism, physiology, and behavior are organized by an endogenous timekeeping system that oscillates with a circadian (*circa*, about, and *dian*, a day) period.

The multitude of interacting elements that generate and coordinate circadian rhythms throughout the body constitute the circadian timing system. Endogenous circadian rhythms are fundamental properties of all cells. They emerge from

Abbreviations: AVP, arginine vasopressin; BDNF, brain-derived neurotrophic factor; CaMKII, calcium-calmodulin dependent protein kinase II; cAMP, cyclic adenosine triphosphate; cGMP, cyclic guanine monophosphate; CICR, calcium-induced calcium release; CREB, cAMP response element-binding protein; DHA, dehydroascorbic acid; ERK, extracellular signal-regulated kinase; GABA, gamma-amino butyric acid; GAD, glutamic acid decarboxylase, GC, guanylate cyclase; GRP, gastrin-releasing peptide; GSH, glutathione; GSSH, glutathione disulphide; ipRGCs, intrinsically photoreceptive retinal ganglion cells; JNK, c-jun kinase; K2P, two-pore domain potassium channel; KCNK, potassium channel subfamily K; LTD, long-term depression; LTP, long-term potentiation; MAPKK/MEK, MAPK kinase; MAPKs, mitogen activated protein kinases; NMDA/ NMDA•R, N-methyl D-aspartate/ N-methyl D-aspartate receptor; NO, nitric oxide; NOS, nitric oxide synthase; NPAS2, neuronal PAS-domain protein 2; ODNs, oligodeoxynucleotides; PACAP, pituitary adenylate cyclase-activating peptide; PKA, cAMP-dependent protein kinase; PKG, cGMP-dependent protein kinase; PVN, paraventricular nucleus; RHT, retinohypothalamic tract; ROR, retinoic acid-related orphan receptor; RXO, reduction-oxidation oscillator; RyR, ryanodine receptor; SCN, suprachiasmatic nucleus; TASK, TWIK-related acid-sensitive K^+ channel; tPA, tissue-type plasminogen activator; TREK, TWIK-related K^+ channel; TrkB, tropomyosin-related receptor kinase; TTO, transcription-translation oscillator; TTX, tetrodotoxin; TWIK, Tandem of P domains in a Weak Inward rectifying K^+ channel; VIP, vasoactive intestinal peptide; VIP•R (also VPAC•R), vasoactive intestinal peptide receptor; V_m, membrane potential.

[1]This paper is dedicated to the memory of our friend and colleague, William T. Greenough, a pioneer in the discovery of brain plasticity.

self-regenerative oscillations in transcription-translation of core timing (clock) genes, gene products, and their post-translational modification and in cellular metabolism of reduction-oxidation (redox) states. Cellular circadian rhythms of various tissues, e.g., heart, lung, blood, and brain, are synchronized by the hypothalamic suprachiasmatic nucleus (SCN) (Lehman et al., 1987; Ralph et al., 1990; Yoo et al., 2004). The SCN coordinates the myriad body clocks via diverse output signals and adjusts its circadian state via inputs that communicate desynchronization with environmental and internal conditions. Light-stimulated resetting of SCN circadian phase is a form of neuronal plasticity, *circadian plasticity*, mediated by cellular processes similar to those that cause long-term changes in hippocampal state, but susceptibility is temporally restricted to night.

As understanding of mechanisms that lead to memory formation in the hippocampus has grown, questions have arisen about how such plasticity is regulated to prevent network hyperstimulation, saturation, and impaired recall and cognition. This led to the prediction and substantiation of metaplastic regulatory mechanisms. *Metaplasticity* is a persistent change in the state of synapses or neurons due to past activity or modulation that alters responses to subsequent plasticity-inducing stimuli. Consequently, responses may be altered in amplitude or duration compared with the basal response (Abraham and Bear, 1996). Metaplastic modulation can be local, involving single or nearby synapses, or extend throughout the neuron or network (Hulme et al., 2014). Behavioral expression of metaplasticity includes responses to environmental stimuli (enrichment or stress), developmental deprivation (visual or tactile stimulation), and changes due to associative learning (Abraham, 2008).

In this review, we consider the commonality of mediators of plasticity effectors and mechanisms between LTP in the hippocampus and circadian plasticity in the SCN. Once initiated, these feed-forward processes involving excitability and intracellular networks transform these systems into new, persistent states. On a higher-order level, the ability of the SCN to express plasticity-induced state changes is dependent upon the context that is modulated by the cycling circadian clock and the associated state changes in SCN neurons. This susceptibility to light-induced plasticity returns each night, as the circadian clock repeats its daily cycle, a phenomenon we term *iterative metaplasticity*. We propose that the hippocampal circadian clock similarly modulates the potential for LTP and other forms of synaptic plasticity by iterative metaplasticity.

WHY ARE THERE INTERNAL CIRCADIAN SYSTEMS?

The internal milieu is not resting in a basal state when stimuli are encountered. Rather, the internal environment—from the level of cells to tissues, organs, and brain and body systems–oscillates with a major regular periodicity near 24 h, as well as various ultradian rhythms. Consequently, patterning of behavioral outputs changes significantly over this period, so that some behaviors occur in the day, others at night, and some are expressed at dawn and dusk.

Daily internal oscillations are a result of adaptation to a major environmental variable over the course of evolution: the ever-changing cycle of day and night generated by the Earth's rotation on its axis. Early organisms that optimized cycles of behavior to adapt to these changes would have held a competitive advantage (Ouyang et al., 1998; Woelfle et al., 2004; Gerstner, 2012). Indeed, selection for mechanisms that internalized timekeeping embedded circadian clocks within genomes and basal metabolism (Lowrey and Takahashi, 2011; O'Neill and Reddy, 2011; O'Neill et al., 2011; Gillette and Wang, 2014). This encoded the patterning of environmental state within genes of some prokaryotes and all eukaryotes, enabling them to organize day vs. nighttime processes internally and predict these environmental state changes.

Rhythmic behaviors that anticipate rather than merely react to environmental changes would offer significant benefits. Functional and physiological changes can be initiated *in advance* of environmental changes, optimizing behavior and aligning it more closely to daily environmental cycles. Further, because day-night durations undergo seasonal variations, an anticipatory, endogenous rhythm generator would adapt and entrain to changing ratios of light: dark periods, generating seasonal plasticity in behaviors (Bartness et al., 1993; Ebling et al., 1995; Nuesslein-Hildesheim et al., 2000; Meijer et al., 2010).

Anticipatory, near-24-h *circadian rhythms* are generated by molecular and cellular processes, and result in appropriately timed cycles of physiology, metabolism, and behavior. They persist in constant darkness. The endogenous period of circadian clocks under these aperiodic conditions is close-to, but not exactly, 24-h (King and Takahashi, 2000; Okamura et al., 2002). Consequently, *free-running* rhythms drift out of correspondence with day and night of the solar cycle. They re-adjust in response to light signals that occur during the subjective night of circadian clock processes. Thus, when animals are exposed to regular 24-h cycles of light and dark or light presented briefly at regular 24-h intervals, the rhythms undergo *entrainment*, aligning with the time-schedule dictated by light (Golombek and Rosenstein, 2010). Because light can communicate environmental timing to entrain the circadian system, it is a *zeitgeiber* (time-giver).

CIRCADIAN RHYTHMS ARE GENERATED BY MOLECULAR AND METABOLIC OSCILLATORS

How do molecules and metabolism generate self-sustaining near 24-h oscillators? The endogenous timing mechanism consists of a transcription-translation oscillator (TTO) with negative feedback (Lowrey and Takahashi, 2011) that interacts with a reduction-oxidation oscillator (RXO) (Gillette and Wang, 2014). In the TTO, heterodimers of positive transcription factors CLOCK/BMAL (or CLOCK/NPAS2 in some brain regions) bind to E-box motifs in the promoters of *Per 1/2/3*, *Cry 1/2*, and *Rev-Erbα*, activating transcription. The protein products undergo complex post-translational modifications. Under appropriate conditions, PER and CRY proteins heterodimerize, translocate to the nucleus, and repress transcriptional activation by CLOCK/BMAL. An additional regulatory feedback loop involves the negative regulator, REV-ERBα (a nuclear heme receptor) and the positive regulator, retinoic acid-related orphan receptor (ROR) (Lowrey and Takahashi, 2004; Yin et al., 2007, 2010). These antagonistic regulators compete for binding to ROR elements (ROREs) within the promoters of *Bmal1* and *CLOCK*. REV-ERBα also can modulate transcription by binding to ROREs in the *Per* and *Cry*

promoters. Transcription-factor binding is a dynamic process, which permits regulation based on relative amounts and states. Rates of synthesis and proteasomal degradation of clock proteins are important to rhythm generation (Gillette and Mitchell, 2002; Nitabach et al., 2002; Lundkvist et al., 2005; Golombek and Rosenstein, 2010; Van Ooijen et al., 2011). When PER and CRY are ubiquitinated and degraded, the cycle of *Per* and *Cry* transcription-translation repeats. The TTO takes ∼24-h to complete one cycle, as does an accessory loop comprising the transcription factor REV-ERBα. REV-ERBα binds to ROREs in promoter regions of the *Clock* and *Bmal1* genes, initiating their transcription.

The RXO emerges from robust, near-24-h rhythms of cellular metabolic state. Redox state oscillates in SCN samples *ex vivo*, as well as brain slices *in vitro*. This RXO modulates the membrane potential (V_m) of SCN neurons and thus the state of excitability (see further). Drivers of the RXO are presently unknown, however, redox oscillations are wide spread in circadian systems (O'Neill and Reddy, 2011; O'Neill et al., 2011). Transcriptional regulation via REV-ERBα, an endogenous heme receptor, is exquisitely sensitive to cellular redox state. This is one of several nodes of interaction between the TTO and the RXO (Gillette and Wang, 2014). The interacting oscillators generate rhythms in the synthesis of key cellular proteins, firing rate of neurons, and release of neuropeptides (Hatcher et al., 2008; Wang et al., 2012). These clock-controlled rhythms can have distinct phase-relationships, patterns, and amplitudes. Time-of-day restrictions on susceptibility to phase-resetting signals emerge from the complexity of theses dynamic systems.

CIRCADIAN OSCILLATION IN SCN EXCITABILITY

The daily rhythm of electrical activity in SCN neurons is essential for the central pacemaker to synchronize circadian clocks throughout the body to each other and to changing environmental time cues (Brown and Piggins, 2007; Colwell, 2011). SCN neurons exhibit a daily fluctuation of spontaneous action potentials (SAP), with higher frequency during the daytime than the night. The SAPs are autonomously generated by the SCN (Brown and Piggins, 2007), and their patterned behavior can be detected both *in vivo* and *in vitro*, by single- or multi-unit recording (Inouye and Kawamura, 1979; Green and Gillette, 1982; Welsh et al., 1995). Dissection of underlying ionic mechanisms by patch-clamp recordings of membrane properties of SCN neurons from mouse (Belle et al., 2009) and rat (Wang et al., 2012) reveals at least three ionic factors, K^+ and Ca^{2+} currents and $[Ca_i^{2+}]$, underlie rhythmic oscillating membrane potential (V_m) (Belle et al., 2009). These ionic features represent three functional categories (Colwell, 2011): (1) currents *providing* the excitatory drive that elevates V_m to the threshold of action-potential generation; (2) currents *responding* to the excitatory drive and generating action potentials; and (3) currents contributing to the nightly silencing of firing through hyperpolarization of the membrane. Modulation of these currents could be on the levels of channel expression, localization, post-translational modification of conductance, and/or gating properties. Circadian oscillation in V_m is necessary for timekeeping. Electrical silencing of pacemaker neurons in *Drosophila* by genetic manipulation of K^+ channels stops the free-running circadian clock, resulting in arrhythmic behavior (Nitabach et al., 2002).

While links between oscillations in V_m and circadian plasticity have not been fully established, recent studies suggest important roles for the neuropeptides released in the SCN. Their circadian rhythm is driven by oscillation in V_m, which fine-tunes the neuronal responses to input signals. Among the more than 200 neuropeptides identified in SCN, vasoactive intestinal peptide (VIP) is the most characterized and well studied. VIP is an essential neuropeptide for the synchrony of the brain clock (Aton et al., 2005). VIP binds to a G-protein-coupled receptor (VIP•R 2), activating cAMP and PKA pathways (Hao et al., 2006). cAMP is essential for the maintenance of intrinsic circadian rhythmicity; it is also important in information processing within the brain clock (Hastings et al., 2008). cAMP was the first confirmed non-transcriptional cytosolic oscillator in SCN neurons (O'Neill et al., 2008), followed by Ca^{2+} (Harrisingh et al., 2007), PKC (Robles et al., 2010), small G protein (Brancaccio et al., 2013), and other small molecules (Dodd et al., 2007).

Beyond VIP, numerous neuropeptides have been found to be important to regulating clock function. Examples include the following. **(1)** Mice lacking arginine vasopressin (AVP) receptors are more resistant to jet-lag and their recovery period is more rapid than wildtypes (Yamaguchi et al., 2013). This feature results from the looser coupling between cells in AVP receptor-deficient SCN, which maintains the basic circadian rhythm under steady state, but is more responsive to *zeitgebers* for extreme phase-shift. **(2)** Gastrin-releasing peptide (GRP) is another major neuropeptide in the brain clock. Activation of GRP-receptors produces long-lasting excitation in SCN neurons (Gamble et al., 2007). This response is stronger in subjective night than daytime. The underlying mechanism involves inhibition of resting K^+ current and subsequent membrane depolarization (Gamble et al., 2011). **(3)** Pituitary adenylate cyclase-activating peptide (PACAP) is present in retinohypothalamic tract (RHT) terminals that invest SCN tissue. PACAP alters the excitability of SCN neurons in a bimodal manner: it suppresses the discharge of some of the neurons via VIP•R2 receptors, while excites others via VIP•R1 receptors (Reed et al., 2002). PACAP also modulates light-induced phase-shift in complex ways: it facilitates phase-delay but attenuates phase-advance; the underlying mechanism has yet to be revealed (Chen et al., 1999).

The SCN releases these, and many other neuropeptides, with levels of release following a circadian pattern (Hatcher et al., 2008). It is thought that these neuropeptides also play a role in synchronizing clocks in other brain regions by carrying time-of-day information from the SCN (Lee et al., 2010). It follows that neuropeptides may contribute to clock-based metaplasticity.

THE MANY BRAIN CONNECTIONS OF THE SCN

The SCN has direct and indirect connections with many brain sites. Recent assessments identify 35 brain regions projecting directly to the SCN, and if multi-synaptic inputs are included, this number increases to 85 projecting regions (Morin et al., 2006; Morin, 2013). Input from the retina via the retinohypothalamic tract (RHT), the thalamic intergeniculate nucleus via the geniculohypothalamic tract (GHT) and the median raphae nucleus are

considered critical to the "circadian visual system" (Morin and Allen, 2006). A study of the various primary and secondary inputs conducted using retrograde tracers (Krout et al., 2002) found many sources for SCN afferents, including regions in the hippocampal formation. Many brain regions are targets for efferent projections from the SCN (Abrahamson and Moore, 2001; Morin, 2013). Of particular interest is the indirect connection of the SCN, via the dorsomedial hypothalamus, to the locus coeruleus (Aston-Jones et al., 2001; Markov and Goldman, 2006), a region known to mediate cortical and hippocampal activation (Berridge and Foote, 1991).

CIRCADIAN CLOCKS IN THE BRAIN

The SCN is acknowledged to be *the* central circadian clock in mammals (Lehman et al., 1987; Ralph et al., 1990). The SCN maintains its autonomous circadian rhythm when surgically isolated from the rest of the brain *in vivo* (Inouye and Kawamura, 1979) and *in vitro*, where synchronized near-24-h rhythms of electrical activity, metabolism, and clock-gene expression persist (Schwartz et al., 1980; Gillette and Prosser, 1988; Prosser et al., 1989; Yamazaki et al., 1998; Wang et al., 2012). Whereas all cells of the body possess intrinsic clocks, in the absence of the SCN the myriad cellular TTOs drift out of phase with one another. This was demonstrated elegantly in a mouse bearing a transgene reporter, PER 2::LUCIFERASE, where circadian rhythms in this clock protein are expressed as bioluminescence and can be measured non-invasively in all cells and tissues (Yoo et al., 2004). When tissues were assessed *in vivo* after SCN lesion or cultured in isolation *in vitro,* individual cells from all tissues examined continued to exhibit circadian oscillations, but with a range of phases that appeared arrhythmic when averaged (Welsh et al., 1995; Yoo et al., 2004).

Whereas the rest-activity cycles of nocturnal and diurnal animals are in anti-phase, SCN electrical activity in nocturnal and diurnal animals alike peaks during the light phase (Fuller et al., 2006; Brown and Piggins, 2007). This indicates the switch in peak activity in other brain regions that drive the behavioral differences between nocturnal and diurnal animals lies downstream of the SCN, and affirms the autonomy of SCN electrical activity rhythms. Extra-SCN brain tissue, such as the paraventricular nucleus (PVN), drifts out of synchrony *in vitro* but rapidly re-synchronizes to the rhythm of the SCN in co-culture (Tousson and Meissl, 2004). Mediators of this inter-region synchronization are subjects of current study. Multiple modes of communication of phase have been identified, including the electrical communication via neuronal circuits, as well as via diffusible signals (Silver et al., 1996; Tousson and Meissl, 2004; Guo et al., 2005; Kalsbeek et al., 2006; Welsh et al., 2010). Thus, in each cell of the body an endogenous circadian clock controls the daily timing of cell-specific transcription, cell dynamics, and signaling, but it relies on information from the SCN for coordination.

In addition to its autonomous ∼24-h rhythmicity, the SCN holds a privileged position among circadian clocks at the cell and systems levels in receiving direct information about the presence and intensity of environmental light from the retina via the RHT. It is then able to transmit this information to peripheral clocks. Intrinsically photoreceptive retinal ganglion cells (ipRGCs) act as photon-counters, marking the presence, duration, and intensity of light (Berson et al., 2002; Hattar et al., 2002). Their axons innervate the SCN and communicate the presence of light by releasing glutamate and the co-localized peptide, PACAP, onto the ventral SCN (Chen et al., 1999). The SCN integrates these signals and transmits information about the environmental light profile to all other cellular circadian clocks in the brain and body. The SCN also receives timing signals from other, less potent, *zeitgeibers* including locomotor activity, sleep/wake states and nutritional status (Stephan, 1989; Lamont et al., 2005; Fuller et al., 2006), resulting in feedback control of the circadian timing system.

Circadian rhythms of clock genes have been reported in several brain regions, including the prefrontal cortex, olfactory bulb, and hippocampus (Abe et al., 2002; Granados-Fuentes et al., 2006; Li et al., 2013). The hippocampus exhibits circadian oscillation in the expression of *Per2,* a hallmark of the TTO. The amplitude and persistence of LTP in the CA1 region varies in a circadian manner (Chaudhury and Colwell, 2002; Chaudhury et al., 2005). Mutations in *Per 2* that impair the circadian clock result in abnormal hippocampal LTP (Wang et al., 2009). This supports a necessary role for the circadian clock in permitting and enabling hippocampal plasticity.

CIRCADIAN OSCILLATION IN SUSCEPTIBILITY TO LIGHT-INDUCED PLASTICITY

The SCN processes information about significant variations in availability of light or nutrients that necessitate adjustment of circadian timing. It dynamically responds to cues that communicate mismatch between internal and environmental time. Light, the signal of day-length, alters clock gene expression, as well as phases of the circadian oscillations in heart rate, ingestion, and wheel-running behavior.

The SCN has the unusual property of responding to light differently at different points in the circadian cycle (**Figure 1**). The phase of rhythms in clock gene expression, neuronal firing, and locomotor activity are unaffected by light in the daytime, but change significantly when light is encountered during nighttime. The response to nocturnal light is bifurcated (Ding et al., 1994). Light in the early night (the period after the normal light-off) signals a prolonged day; the clock responds by delaying its phase. In the late night (the period before lights-on), however, light signals an early dawn, advancing clock phase prematurely to a daytime state. The SCN generates such differential responses by selectively *gating* its susceptibility to inputs (see further). Gating is regulated at multiple levels, including neurotransmitter receptors and effectors of intracellular signaling pathways (Gillette and Mitchell, 2002; Golombek and Rosenstein, 2010).

Light is communicated to the SCN from the retina by glutamatergic neurotransmission from the RHT (Ding et al., 1994; Welsh et al., 2010). During subjective nighttime, glutamate or the agonist N-methyl D-aspartate (NMDA), are sufficient to activate light signaling *in vivo* (Colwell and Menaker, 1992; Vindlacheruvu et al., 1992; Gannon and Rea, 1993, 1994) or the SCN brain slice *in vitro* (Ding et al., 1994; Shirakawa and Moore, 1994). They mimic the effects of light: changing circadian phasing, inducing *c-fos* and other immediate early genes (Ebling, 1996;

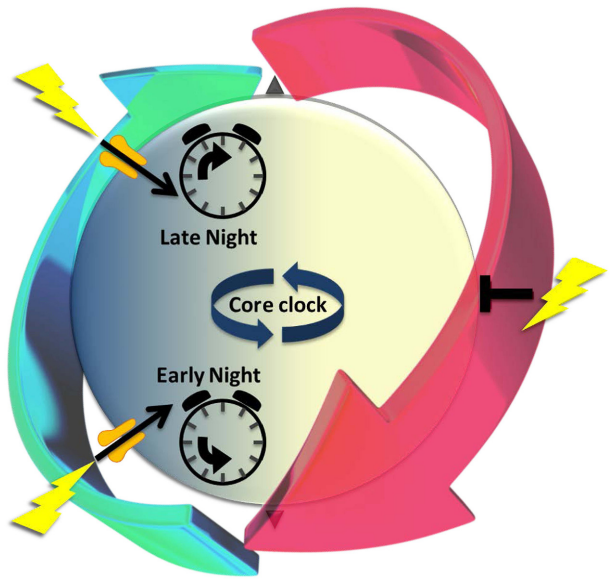

FIGURE 1 | The cyclic series of dynamic cellular states of the endogenous circadian clock is characterized by changing susceptibility that recurs every ~24 h to signals that alter clock state, a paradigm of *iterative metaplasticity*. The periodic recurrence of night and day provides an oscillatory environmental context for life on Earth. A near-24-h dynamic series of functional states organizes differential responses to light that depend upon time-of-day. Light-driven glutamatergic signals (lightning bolts) at night (blue) alter suprachiasmatic nucleus (SCN) state-dynamics and outputs; daytime (yellow) stimulation is without effect. Circadian timekeeping mechanisms generate these windows of susceptibility, poising the SCN to respond appropriately to a temporal error signal- in early night moving clock state back to an earlier time or in late night advancing it prematurely toward morning. These long-lasting changes in clock state express the hallmarks of neuronal plasticity. The gate to light-signaling is open transiently during nighttime (green arrow), but closed in daytime (red arrow). The gating mechanisms permissive for state changes are clock-driven, preceding the light signal. Thus, light-induced plasticity occurs only if the functional state of cells is permissive at that time. Underlying differences in susceptibility are cyclic states of excitability and intracellular signaling elements that prime SCN receptivity to plasticity signals. We propose that this gating of light-signaling responsiveness, which cycles over the night and day, is a paradigm of *iterative metaplasticity*, the repeated, anticipatory susceptibility to induction of neuronal plasticity.

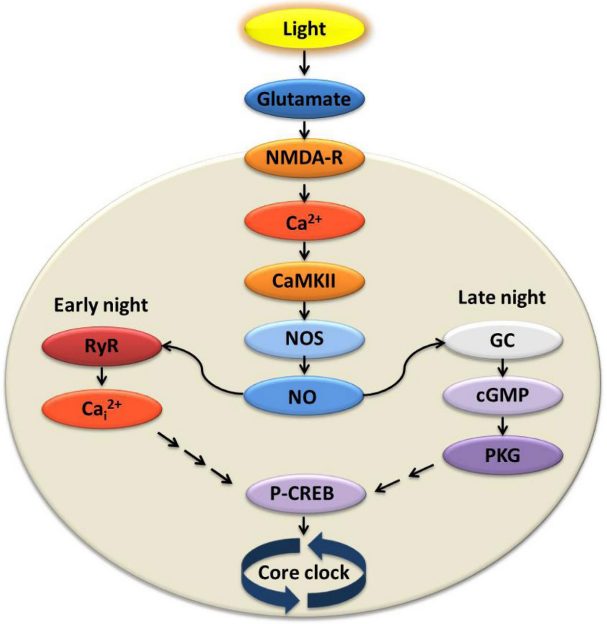

FIGURE 2 | Signal transduction at the suprachiasmatic nucleus (SCN) in response to light activates divergent pathways in early vs. late night. Light experienced at night transmits signals via the retinohypothalamic tract (RHT) to the SCN causing glutamate release. Glutamatergic activation of the NMDA receptor is necessary and sufficient for initiating state changes, and leads to influx of extracellular Ca^{2+}. Ca^{2+}/calmodulin-dependent kinase II (CaMKII) and nitric oxide synthase (NOS) are activated, increasing levels of nitric oxide (NO). In the early night, the rise of NO activates ryanodine receptors (RyR) on the intracellular endoplasmic reticulum where Ca^{2+} is stored. Intracellular Ca^{2+} (Ca_i^{2+}) is released via the activated RyR and, through a mechanism yet to be elucidated, leads to phosphorylation of cAMP response element-binding protein (pCREB) and subsequent increased expression of clock genes. During the late night, however, NO activates guanylyl cyclase (GC), cGMP synthesis, and increased activity of cGMP-dependent protein kinase (PKG). Activation of this and other kinases again leads to increased pCREB and transcription of key clock genes. This simplified model includes only those elements necessary and sufficient to stimulate state changes similar in amplitude and timing to light-induced responses.

Guido et al., 1999) and the clock gene *Per 1* (Moriya et al., 2000). NMDA receptor antagonist application effectively blocks light-induced changes. In subjective daytime, glutamate exposure does not activate these signaling pathways, nor does it cause a change in phase.

Initial steps of glutamate signaling include NMDA receptor (NMDA•R) activation, Ca^{2+} influx into cells in the SCN (Ding et al., 1998; Obrietan et al., 1998; Colwell, 2000, 2001), and stimulation of downstream kinases (**Figure 2**). Among the earliest changes is in Ca^{2+}/calmodulin-dependent kinase Type II (CaMKII), which is activated by auto-phosphorylation. Inhibition of CaMKII in early night blocks light-induced phase delays and changes in *c-fos* and *Per 1/Per 2* expression in the SCN (Golombek and Ralph, 1995; Fukushima et al., 1997; Yokota et al., 2001). Active pCaMKII phosphorylates neuronal nitric oxide synthase (nNOS) (Agostino et al., 2004), triggering nitric oxide (NO) production. NOS activity is necessary for light-induced plasticity in the SCN (Ding et al., 1994, 1997; Melo et al., 1997), and the response to light or glutamate increases in the presence of an NO donor (Melo et al., 1997).

The light signaling pathway bifurcates in early vs. late night, downstream of NO (**Figure 2**). During the *early night*, glutamate-induced plasticity requires Ca^{2+}-induced-Ca^{2+} release (CICR) from neuronal ryanodine receptors (RyRs). When RyRs are pharmacologically activated, the effects of light/glutamate in the early night are reproduced. The same agents have no effect in late night or daytime. Further, inhibition of RyRs blocks light-/glutamate-induced phase delay in the early night both *in vivo* and *in vitro*, but has no effect in late night or daytime (Ding et al., 1998).

During *late night*, on the other hand, NO activates guanylyl cyclase (GC), which increases cGMP levels. This in turn leads to the activation of cGMP-dependent kinase (PKG) (Weber et al.,

1995; Ding et al., 1998; Tischkau et al., 2003b). Phase advance caused by light/glutamate in the late night is mimicked by cGMP analogs (Prosser et al., 1989) and blocked by pharmacological inhibition of PKG (Weber et al., 1995; Mathur et al., 1996; Ding et al., 1998; Ferreyra and Golombek, 2001). The same inhibition does not impact the phase delay induced in early night.

In both pathways, downstream from the steps described, there is transient and rapid phosphorylation of the Ca^{2+}-cAMP response element-binding protein (CREB), leading to transcription of *Per 1,* as well as other CRE-mediated genes (Ginty et al., 1993; Ding et al., 1997; Gau et al., 2002). Phase advance during late night is blocked by antisense oligodeoxynucleotides (αODNs) of CRE sequences. These Ca^{2+}/cAMP response element decoys (CRE-decoys) sequester pCREB and selectively block the advance in clock phase (Tischkau et al., 2003a).

Other factors downstream of glutamate-induced signaling in the SCN are the mitogen-activated protein kinases (MAPKs) and cAMP-dependent kinase (PKA). Both light-induced phase delays and advances are partially blocked by inhibitors of p44 MAPK/ERK1 and MAPK kinase (MAPKK/MEK) transcription (Obrietan et al., 1998; Tischkau et al., 2000; Butcher et al., 2002; Antoun et al., 2012). Light pulses during the subjective night induce activation of members of all three MAP kinase pathways: ERK, JNK, and p38 (Butcher et al., 2003; Pizzio et al., 2003). Signaling via p44/42 MAPK (ERK1/ERK2) is necessary for Ca^{2+}-induced CRE-mediated gene transcription (Obrietan et al., 1998; Butcher et al., 2002; Antoun et al., 2012). PKA activation by light and glutamate has differential effects on SCN state at early *vs.* late night. Upon light or glutamate stimulation, PACAP and PKA enhance the amplitude of light-/glutamate-induced phase delay in early night, and diminish the effect in late night (Tischkau et al., 2000). These effects are like those of PACAP, which were described earlier.

Various regulators of light-induced plasticity affect the network properties of SCN neurons. Brain-derived neurotrophic factor (BDNF) is expressed in the SCN. The cognate receptor of BDNF, tropomyosin-related receptor kinase B (TrkB), is expressed in the fibers of the RHT (Allen and Earnest, 2005). BDNF expression and release are rapidly increased by neuronal activity, which regulates presynaptic release and direct activation of membrane channels (Rose et al., 2004; Blum and Konnerth, 2005). This enhanced expression and response to optic nerve stimulation suggest a role for BDNF in modulating photic input to the SCN (Allen and Earnest, 2005). BDNF- and TrkB-deficient mice exhibit a reduction in the phase-shifting effects of light on the circadian system (Liang et al., 2000). BDNF cannot shift the phase of the clock itself (Prosser et al., 2008; Mou et al., 2009), but interfering with BDNF signaling in the SCN blocks or strongly inhibits phase shifts induced by light and glutamate in the subjective night (Liang et al., 2000; Michel et al., 2006). SCN treatment with either BDNF or tissue-type plasminogen activator (tPA) permits light and/or glutamate to induce phase shifts in the daytime (Liang et al., 2000; Mou et al., 2009). BDNF thus contributes to *gating* of SCN responsiveness to state-changing signals, a concept we will explore in the next section. Gating of the light-response in the SCN is conferred by regulaton of glutamatergic synaptic transmission in the SCN (Kim et al., 2006).

Time-of-day-dependent neurotransmission via the inhibitory neurotransmitter gamma-amino butyric acid (GABA) profoundly affects SCN responses to light. GABA and its biosynthetic enzyme, glutamatic acid decarboxylate (GAD), are widely expressed in SCN neurons (Card and Moore, 1984; van den Pol, 1986; Okamura et al., 1989; Moore and Speh, 1993). Both $GABA_A$and $GABA_B$ receptors have been localized within the SCN by binding and functional studies (Francois-Bellan et al., 1989; Liou et al., 1990; Mason et al., 1991). Further, both GAD activity and GABA levels undergo circadian oscillations in the rat SCN (Aguilar-Roblero et al., 1993). Modulatory effects of GABA signaling on light-induced plasticity are complex, and consistent with a model wherein $GABA_B$ receptors regulate photic signals via presynaptically modulating glutamatergic input from the RHT, whereas $GABA_A$ receptors are proposed to act in the photic signal transduction cascade in local circuits downstream of glutamatergic inputs (Ralph and Menaker, 1989; Gillespie et al., 1997). GABAergic activation of $GABA_B$ receptors diminishes release of labeled glutamate at the SCN in response to optic nerve stimulation (Liou et al., 1986). Consistent with this observation, $GABA_B$ receptor agonists reduce light-induced phase delays in early night; an antagoinist of $GABA_B$ receptors enhances this phase delay (Gillespie et al., 1997). With regard to light-effects in late night, microinjections of agonists of either $GABA_A$and $GABA_B$ receptor agonists into the SCN region significantly diminish phase-advances. Thus, GABAergic modulation of glutamatergic and downstream synapses contributes to gating and amplitude of the light-response in the SCN. Inhibition of the phase shift has been proposed to be mediated by the opposing effects of light and GABA on *Period* gene expression (Ehlen et al., 2008). Additionally, GABA modulates light-induced plasticity by inducing divergent responses in intracellular Ca^{2+} mobilization in SCN neurons (Irwin and Allen, 2009).

Although the complete signaling cascade from light to clock gene expression remains unresolved, the roles of these well-studied effectors downstream of light/glutamate provide insights into the most completely studied form of *circadian plasticity* in the SCN (**Figure 2**). The elucidation of the roles of several of these effectors in SCN plasticity, including pCREB and RyRs, was influenced by their role in plasticity signaling in the hippocampus (Ding et al., 1994, 1997, 1998).

CIRCADIAN GATING OF CLOCK FUNCTION: ITERATIVE METAPLASTICITY

Because SCN responsiveness to light-induced state changes waxes and wanes with each daily circadian cycle, states of sensitivity to plastic change repeat each night, when light has the potential to interrupt the anticipated darkness. Thus, the nocturnal state of SCN activity alters the magnitude and/or duration of plastic events compared with the daytime state. Dynamic linkage of temporal history and state with current responsiveness defines *metaplasticity* (Abraham and Bear, 1996). At night, the SCN responds to light as an error signal–it infers a mismatch between the timing of the clock and the environmental light cycle. The SCN must be able to distinguish *nocturnal* light as an error signal to respond with temporally appropriate phase-resetting. The restricted, time-of-day dependence of susceptibility of the

SCN to light establishes conditions for the *iterative metaplastic state*.

During the night, the manner in which phase is affected varies depending on proximity of the signal to the subjective dusk or dawn encoded in the circadian clock. Dynamic *gating* of inputs to the SCN ensures that the same input (light) has restricted and differential effects on output that depend on the time of day during stimulation. Gating is achieved by clock-driven rhythms in V_m, expression of plasticity modulators, and expression/receptiveness of intracellular effectors of plasticity. This implies the presence of a reciprocal relationship wherein various inputs alter timekeeping elements and induce plasticity, and their oscillations are in turn controlled by the circadian clock. Circadian control of input allows the clock to prime SCN neurons in anticipation of the activation of specific signaling cascades at specific times of the day. Key signaling elements that are potential targets of metaplastic regulation follow.

MEMBRANE EXCITABILITY

The proposed model is that SCN neurons are highly electrically active during daytime, preventing additional excitatory input from the RHT from inducing further changes. At night, however, the neurons are hyperpolarized, thus excitatory input from the RHT elicits significant responses. Supporting this hypothesis, up-regulation of K^+ currents and the consequential hyperpolarized status of V_m during nighttime are essential for gating of phase-shift responses. In excitable cells, V_m is mainly set by a class of two-pore domain K^+ channels (K2P, TASK, and TREK channels) (Hallie, 2001). Gating of these channels is independent of voltage across the membrane; the current is termed leak or background current. K2P channels are encoded by the KCNK gene family. Both *Kcnk1* and *Kcnk2* are expressed in the SCN (Lein et al., 2007); in particular, *Kcnk1* exhibits a robust rhythm in the SCN (Panda et al., 2002). Interestingly, a functional study suggests that the K^+ leak current is regulated by metabolic oscillation in SCN neurons, which provides a non-transcriptional pathway of the clockwork machinery to modulate the membrane excitability (Wang et al., 2012).

INTRACELLULAR Ca^{2+}

Membrane excitability influences neuronal plasticity through intracellular signal pathways where Ca^{2+} plays an essential role. SCN neurons exhibit spontaneous oscillation of intracellular Ca^{2+} (Ca_i^{2+}) concentration, with peak levels during the daytime (Ikeda et al., 2003). While Ca_i^{2+} oscillation is not completely driven by membrane events, the action potential-induced opening of voltage-gated Ca^{2+} channels (L-type) is a proven source of Ca_i^{2+} elevation during the daytime (Irwin and Allen, 2007).

NMDA RECEPTOR

The SCN exhibits circadian expression of mRNAs for subunits of the NMDA receptor (NMDA●R) subunits ε3 and ζ1 in rats; mRNA levels are high during daytime and low during nighttime, whereas protein levels change in anti-phase. Expression of these mRNAs increases in response to light stimulation in the subjective night (Ishida et al., 1994). Both expression and phosphorylation of the ε2/NR2B subunit of NMDA●R undergo circadian variation in hamsters. NR2B mRNA level is high from late day through early night, with the phosphorylated protein peaking in the late night (Wang et al., 2008). Changes in phosphorylation of NMDA●R subunits can correspond to changes in functional properties. An endogenous daily rhythm in the magnitude and duration of NMDA●R-induced Ca^{2+} transients in rat SCN neurons peaks during the night, as does the rhythm in NMDA●R-evoked currents (Pennartz et al., 2001). Thus, evidence indicates that peaks in mRNA abundance of certain subunits precede peaks in NMDA●R phosphorylation. NMDA function peaks in the night in rats, anti-phase to mRNA abundance. Together, these observations indicate that the clock *primes* the SCN to respond to photic signals in anticipation of encountering light signals at night.

CaMKII

Calmodulin kinase II (CaMKII), which is activated in neurons by elevated Ca_i^{2+}, undergoes circadian changes in autophosphorylation state in hamsters, with peak abundance of pCaMKII occurring during subjective day. Total CaMKII levels in the SCN do not vary. Light pulses during the subjective night rapidly phosphorylate CaMKII, which is necessary for the signaling cascade resulting in circadian plasticity (Agostino et al., 2004).

cAMP/PKA

In constant conditions, the rat SCN exhibits spontaneous oscillations in cAMP levels and cAMP-dependent protein kinase (PKA) activity. The endogenous levels of cAMP peak at the end of day and of night (Prosser and Gillette, 1991). While cAMP levels rise in response to light/glutamate stimulation, application of agonists to cAMP/PKA does not mimic the effect of light/glutamate. However, if cAMP/PKA is activated simultaneously with light/glutamate stimulation, resultant plasticity is enhanced in early night, and diminished in late night. Thus, the cAMP/PKA system toggles responses, changing the state of signaling pathways based on time of activation (Tischkau et al., 2000).

cGMP/PKG

cGMP levels and cGMP-dependent protein kinase (PKG) activity also display spontaneous oscillations in the SCN. Tissue cGMP levels remain relatively constant throughout subjective day and night, with a sharp peak appearing at the time corresponding to end of night. PKG activity is markedly higher at end of subjective night compared to the end of subjective day and the middle of subjective night. Sensitivity of the SCN to light-induced increase in cGMP levels and PKG activity occurs just before these values peak endogenously (Tischkau et al., 2003b). This rise in cGMP/activation of PKG is required for clock dynamics to proceed; if either is blocked, clock state reverts by 3.5 h and then recapitulates the intervening period (Tischkau et al., 2004). Peak PKG activity may signal the transition from night to the daytime state. A light-pulse at late subjective night may prematurely shift the clock to this state, thus advancing the clock phase to a point it normally transits at the end of the night state of the circadian cycle and that is required for entry into the daytime state.

MAPK

Three members of the mitogen-activated protein kinase (MAPK) family, ERK, JNK, and p38, undergo oscillations in phosphorylation in hamsters, with peak phosphorylation levels during the day. All three are phosphorylated in response to light at mid-subjective night (Pizzio et al., 2003). In mice, ERK1 and ERK2 signaling is induced by light pulse in the subjective night. Levels of phosphorylated ERK (pERK) show a circadian oscillation, with peak levels at mid-to late-subjective day (Obrietan et al., 1998).

CREB

Phosphorylation of the Ca^{2+}-cAMP response element-binding protein (forming pCREB) links stimulation of these intracellular signaling pathways to transcriptional activation of genes whose promoters bear the Ca^{2+}-cAMP response element (CRE). An endogenous oscillation in basal levels of pCREB occurs in rat and mouse SCN, with a corresponding oscillation in expression of CRE-mediated genes. pCREB levels peak from the middle to end of subjective night. This is followed by peak expression of CRE-mediated genes, which occurs from late-subjective night to mid-subjective day (Obrietan et al., 1999). Light induces ^{133}Ser pCREB at levels proportionate to light intensity and expression of CRE-mediated genes only at subjective night (Ginty et al., 1993; Ding et al., 1997; Gau et al., 2002). This indicates that there is strict regulation of CRE-mediated induction of gene expression during the circadian cycle, with only the night being favorable for the CREB/CRE-transcription pathway (Ding et al., 1997).

BDNF

BDNF mRNA levels in the SCN peak during the early subjective day, and BDNF protein levels peak during the subjective night (Liang et al., 1998). The expression of this rhythm in BDNF is dependent on a tetrodotoxin (TTX)-sensitive neuronal circuit (Baba et al., 2008). These observations support a role for BDNF in gating circadian responses to nocturnal light.

GABA

There is a diurnal rhythm of activity of GAD, which synthesizes GABA (Aguilar-Roblero et al., 1993). GABA release within the SCN also oscillates (Aton et al., 2006), peaking at the early night (Itri and Colwell, 2003). The peak in GABA release coincides with the timing of the most hyperpolarized state of Vm in SCN neurons (Wang et al., 2012). This release pattern is modulated by VIP signaling (Itri and Colwell, 2003; Itri et al., 2004; Aton et al., 2006).

CONCLUSION

Endogenous rhythms in expression/function of these effectors and modulators are timed to allow temporally specific signaling cascades. Functional states of SCN cells with respect to these molecules are varied in *advance* of the point in time when the plasticity-inducing event is anticipated, namely light in the early or late night (Gillette and Wang, 2014). The clock thus *primes* cells to respond to plasticity signals in a specific, time-of-day dependent manner.

These intracellular effectors overlap with some of the effectors that contribute to the standard paradigm for *metaplasticity*, the changes in the state of synapses or neurons that impact the amplitude and persistence of *subsequent* instances of plasticity (Abraham, 2008). Circadian rhythms have been implicated previously as a form of metaplasticty (Gerstner and Yin, 2010; Gerstner, 2012). Therefore, we propose, that the alteration of signal responsivity of SCN cells is a metaplastic form of modulation of plasticity.

With the core clock driving SCN metaplasticity, SCN neurons can show *cyclic* variation in their metaplastic state. This is distinct from metaplastic regulation as it has been described previously (Abraham and Bear, 1996; Abraham, 2008). Persistence of clock-driven metaplasticity is restricted to a regular, discrete period within the 24-h daily cycle. Metaplastic effects that have been studied previously persist from a few minutes to a few days (Abraham, 2008). Therefore, we propose that the repetitive pattern of circadian neuronal state-changes constitutes a paradigm of *iterative metaplasticity* (**Figure 1**).

COMMONALITIES IN PLASTICITY PATHWAYS: SITES OF CLOCK-DRIVEN METAPLASTICITY IN THE HIPPOCAMPUS

The best-characterized form of activity-dependent synaptic plasticity is long-term potentiation (LTP). Defined as persistent enhancement in synaptic efficacy due to repeated activation of the same synapses, it is widely considered, along with long-term depression (LTD), to be the physiological basis for acquiring new memories and enhancing nascent ones (Malenka and Nicoll, 1999; Lisman, 2003; Cooke and Bliss, 2006). While the molecular basis of LTP is still incompletely determined, the roles of a large number of signaling mechanisms and molecular effectors have been elucidated. Most *in vitro* studies on LTP have been conducted by delivering high frequency stimulation to Schaffer collateral fibers connecting the CA3 and CA1 pyramidal neurons. Glutamatergic synapses were the first investigated for LTP and the well-studied synapses between Schaffer collateral fibers and CA1 pyramidal neurons exhibit LTP mediated by NMDA●R activation (Bliss and Collingridge, 1993; Lisman, 2003). There is, however, synapse-to-synapse heterogeneity in the molecular mechanisms associated with LTP.

Deeper understanding of the signaling mechanisms underlying synaptic plasticity has raised questions as to the manner in which these plasticity mechanisms are modulated. While a number of factors can regulate plasticity at the time of occurrence, activity-dependent processes that modulate plasticity by altering the state of synapses or cells *prior* to plasticity-inducing events are metaplastic. That is, *synaptic metaplasticity* can be considered the *plasticity of synaptic plasticity*. It acts to enhance the salience of subsequent exposure to certain types of stimulation, and prevent saturation of LTP and LTD, which can have negative effects on learning and memory as well as neuronal health (Deisseroth et al., 1995; Philpot et al., 2007; Abraham, 2008; Mockett and Hulme, 2008).

Key to the concept of metaplasticity is that any physiological or biochemical change in the state of the cell or synapse needs to persist beyond the initial activity that triggers the metaplastic changes. This distinguishes the paradigm from direct synaptic regulation, which occurs concurrently with synaptic plasticity. The initial bout of activity, or *priming*, changes the

functional state of the synapse, neuron, and network, and thus its susceptibility to future plasticity-inducing events (Abraham, 2008; Hulme et al., 2014).

There is emerging evidence demonstrating that the time-of-day affects the magnitude and persistence of synaptic plasticity. Several studies have shown circadian variation in the efficacy of LTP (Chaudhury et al., 2005; Nakatsuka and Natsume, 2014). Time-of-day effects may be one mechanism of metaplasticity. How the brain clock interacts with synaptic plasticity to cause circadian variation is a question ripe for deeper investigation. Both direct and indirect interactions can be hypothesized.

In this review, we have detailed effectors of SCN plasticity that are under circadian control. Several of these effectors are also involved in hippocampal LTP, including glutamate, NMDA●R, CaMKII, Ca^{2+}, NO, RyR, cGMP/PKG, cAMP/PKA, MAPK, and CREB (Lu et al., 1999; Lu and Hawkins, 2002; Monfort et al., 2002; Cooke and Bliss, 2006; Irvine et al., 2006; Zorumski and Izumi, 2012). While a detailed description of the role of these molecules in LTP is outside the scope of this review, the commonality of signaling molecules between plasticity-inducing events in the SCN and hippocampal LTP is striking. Further, signaling cascades involving some of these molecules are also common to both processes. The NO-GC-cGMP-PKG pathway, which is critical in late night signaling in the SCN, has also been shown to contribute to late-phase LTP (L-LTP) (Lu et al., 1999; Lu and Hawkins, 2002; Ping and Schafe, 2010). The cAMP-MAPK-CREB pathway is also involved in both signaling processes (Gerstner and Yin, 2010). In both cases, this signaling cascade induces plasticity by targeting CREB. Glutamate-induced NMDA●R activation, Ca^{2+} influx, and CaMKII phosphorylation are critical signals that mediate both hippocampal LTP and SCN state changes (Malenka and Bear, 2004). Further, several of the signaling elements described, including NMDA●R, Ca_i^{2+}, PKC, NO, CaMKII, and MAPK, previously have been hypothesized as sites for metaplastic regulation of synaptic plasticity (Abraham, 2008; Lucchesi et al., 2011; Zorumski and Izumi, 2012). Lastly, hippocampal plasticity is modulated by molecules that are also modulators of SCN plasticity, like BDNF (Bramham and Messaoudi, 2005; Lu et al., 2008; Minichiello, 2009; Schildt et al., 2013) and GABA (Arima-Yoshida et al., 2011; Nakatsuka and Natsume, 2014).

Cycling of the circadian clock results in iterative metaplasticity via regulation of the effectors and modulators of plasticity in the SCN. Might such a relationship also exist between clock cycling and hippocampal plasticity? One indication of such a paradigm comes from a study that showed that activity of MAPK as well as adenylyl cyclase, and levels of cAMP vary in a circadian fashion in the hippocampus. These oscillations parallel Ras activity and phosphorylation of MAPK kinase and CREB. The variations persist under free-running conditions, indicating they are endogenous in nature. These oscillations were shown to impact long-term memory (Eckel-Mahan et al., 2008). A further study showed that oscillations of adenylyl cyclase and MAPK in the hippocampus are dependent upon an intact SCN (Phan et al., 2011).

A role for GABA in circadian rhythms of LTP has been demonstrated, with nighttime disinhibition of a $GABA_A$ network shown to facilitate LTP in the CA1 region of the rat hippocampus (Nakatsuka and Natsume, 2014). Further, the clock gene *Per 2* oscillates in isolated hippocampal slices, indicating the presence of an endogenous clock in the hippocampus. In support of this link, mutations in *Per2* cause abnormal LTP in the hippocampus, mediated by decreased phosphorylation of CREB (Wang et al., 2009).

While the peripheral hippocampal clock may itself drive rhythms in levels/activity of specific molecules, there also may be signals from the core SCN clock that drive or synchronize these rhythms. Signals, such as neuropeptides, hormones, and small molecules, may reach the hippocampus directly, or regulate hippocampal activity indirectly. For instance, signaling from the SCN is known to be critical for rhythmic variation in hormone levels. Several hormones that are known to affect hippocampal LTP, including melatonin and cortisol, are released in circadian patterns controlled by the SCN (Reppert et al., 1981; Gillette and Mitchell, 2002; Chaudhury et al., 2005; Chan and Debono, 2010). This could be one mechanism by which the cycling of the clock can lead to time-of-day changes in LTP. In the SCN itself, glutamate application has been shown to induce LTP of field potentials activated by RHT stimulation in a time-of -day dependent manner. These experiments were performed with SCN slices *in vitro*, indicating that the core clock contributes to these time-of-day dependent changes in LTP (Nisikawa et al., 2002).

We propose that **(1)** the commonality of plasticity elements in the SCN and hippocampus, **(2)** the existence of a circadian clock in the hippocampus that modulates the ability to acquire LTP over ∼24-h, and **(3)** evidence for communication between the SCN and the hippocampus all point to clock-driven, iterative metaplasticity in the hippocampus. Further exploration around-the-day of hippocampal expression and activity of molecules, such as NO, PKG, GABA, that have been discussed in this review will shed more light onto clock driven iterative metaplasticity in the hippocampus. The 12-h-limited duration of the persistence of SCN metaplasticity each 24-h cycle makes it a valuable model to probe how such processes affect neurophysiology and the molecular effectors of synaptic plasticity. Such understanding is also critical to elucidating other, non-circadian mechanisms of metaplasticity.

CONCLUSION

Considering circadian gating of inputs in the context of hippocampal and SCN metaplasticity provides striking insights on the ways in which signaling mechanisms conserved between two brain regions can impact their functional states. As a well studied system with an iterative impact on plasticity, the circadian clock offers a compelling model system for the study of metaplasticity. Conversely, new insights on links between circadian rhythms and synaptic plasticity can positively impact the study of SCN and hippocampal function. Further understanding of the interactions between these two critical brain processes will require that future research in either field is more deeply informed by the distinct methodological considerations of the other. The potential benefits to understanding the substrates and dynamics of cognitive disorders in both cases can be enormous.

Box 1 | Key Concepts

Circadian gating
Restricted sensitivity and response to plasticity signals that depends upon the time of day. Whether, and to what extent, phase is reset by neurotransmitter signals is under the control of the circadian clock, which is able to gate sensitivity by regulating membrane state and the expression and function of various signaling effectors of plasticity.

Circadian plasticity
A persistent change of circadian-clock state in response to significant stimulation during a discrete phase of the ∼24-h cycle. When it occurs inappropriately at night, environmental light can permanently change, or reset, the state of the SCN clock. The clock dynamically controls its own susceptibility by circadian plasticity.

Endogenous circadian rhythms
Autonomous, self-generated near-24-h rhythms at any level organization of life, such as expression/function of proteins, cellular physiology, neuropeptide release, and amplitude of behavior. Circadian rhythms are defined by their ability to persist with near-24-h periods in the absence of exogenous temporal cues. For example, animals maintained in aperiodic environments such as constant darkness and SCN brain slices maintained *in vitro* exhibit various circadian rhythms, ranging from the rhythmic patterning of wakefulness and sleep, neuronal firing rate, neuropeptide release to cellular metabolic state. They are driven at the cellular level by a transcription-translation oscillator (TTO) and a redox oscillator (RXO).

Metaplasticity
A plasticity regulatory phenomenon where experience alters the ability of a system to respond to a subsequent plasticity-inducing stimulus. Metaplasticity was originally described as a mechanism for regulating and tuning synaptic plasticity, but also can alter cell or network state. A critical feature of metaplasticity is that once triggered, metaplastic change must persist long enough to impact a plasticity event occurring at a later time. A metaplastic state can either increase or decrease the amplitude or duration of responses to a later plasticity event.

Iterative Metaplasticity
Gating of receptivity to subsequent signals that repeats on a cyclic timebase. An example is gating of susceptibility to light-induced plasticity by the dynamic of the circadian clock in the SCN. Gating of receptivity is achieved by clock-generated, ∼24-h rhythms in neuronal membrane state and/or expression or activation state of intracellular signaling pathways permissive for light/glutamate stimulation.

AUTHOR CONTRIBUTIONS

Rajashekar Iyer, Tongfei Wang and Martha Gillette wrote the text; Martha Gillette revised the text, and provided expertise and funding.

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Experimental sleep deprivation as a tool to test memory deficits in rodents

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Paradigms of sleep deprivation (SD) and memory testing in rodents (laboratory rats and mice) are here reviewed. The vast majority of these studies have been aimed at understanding the contribution of sleep to cognition, and in particular to memory. Relatively little attention, instead, has been devoted to SD as a challenge to induce a transient memory impairment, and therefore as a tool to test cognitive enhancers in drug discovery. Studies that have accurately described methodological aspects of the SD protocol are first reviewed, followed by procedures to investigate SD-induced impairment of learning and memory consolidation in order to propose SD protocols that could be employed as cognitive challenge. Thus, a platform of knowledge is provided for laboratory protocols that could be used to assess the efficacy of drugs designed to improve memory performance in rodents, including rodent models of neurodegenerative diseases that cause cognitive deficits, and Alzheimer's disease in particular. Issues in the interpretation of such preclinical data and their predictive value for clinical translation are also discussed.

Keywords: sleep, sleep-deprivation, memory, learning, cognitive impairment, Alzheimer's disease, rat, mouse

INTRODUCTION

Everyone is aware of the detrimental effects of insomnia, and hundreds of scientific papers have documented the negative consequences of sleep loss. Experimental procedures of sleep deprivation (SD), both in humans and in animal models, have been widely employed to unveil various aspects of sleep function *per se* as well as to study the effects of sleep loss on subsequent brain function at the molecular, cellular, physiological, and cognitive levels.

The first reported experimental study on SD was conducted on puppies at the end of the 19th century (de Manaceine, 1894), followed by other pioneering reports on experimental animal insomnia, mainly in dogs (for a review see Bentivoglio and Grassi-Zucconi, 1997), and by the first formal human SD study (Patrick and Gilbert, 1896). In the following decades, the dog as animal model for SD was progressively replaced by the cat and later on by rodents, with the rat being the animal of choice up to this day. With the introduction of gene manipulation technology, wild-type and transgenic mice are increasingly used in SD studies.

Just like insomnia, experimental SD causes measurable deficits in a variety of cognitive tasks. These deficits can be used to elicit mild, transient cognitive impairment in an otherwise normal individual. Such challenge can be of translational relevance in preclinical and clinical studies aimed at evaluating the efficacy of symptomatic drugs designed to improve cognitive performance. This is of critical importance for Alzheimer's disease (AD), for which an evaluation of the predictive value of preclinical testing is urgently needed.

Several review papers have summarized evidence on SD-related cognitive impairments in humans (Walker, 2008; Killgore, 2010; Lim and Dinges, 2010), but much less attention has been devoted to comprehensive accounts describing comparable results in animal models. A recent review article describes the consequences of sleep loss on a variety of cognitive tasks in rodents (McCoy and Strecker, 2011). The present review has instead a specific focus on studies in which experimental SD in rodents is followed by assessments of memory functions; special attention is paid to the practical guidelines for the design of experiments in which SD is used to induce a transient memory deficit. Studies on the use of SD in the investigation of the effects of cognitive enhancers are also dealt with.

Rodents have been widely used in sleep research to study sleep architecture, as well as sleep homeostasis, circadian rhythms, and their neurochemical and molecular correlates. The idea to exploit the effects of SD on subsequent cognitive abilities is relatively recent, and the present in-depth analysis focuses on papers that include behavioral testing of animals following SD and on studies that explicitly and accurately describe the methodological aspects of the SD protocol and behavioral paradigm.

Abbreviations: AD, Alzheimer's disease; CS, conditioned stimulus; DOW, disk over water; EEG, electroencephalography; EPM, elevated plus maze; FC, fear conditioning; GH, gentle-handling; L/L, light-light; MCI, mild cognitive impairment; MWM, Morris water maze; NOR, novel object recognition; NREM, non-rapid-eye movement sleep; PM-DAT, plus maze discriminative avoidance task; RAM, radial-arm maze; RAWM, radial-arm water maze; REM, rapid-eye movement; SD, sleep-deprivation; SS SD, state-selective sleep-deprivation; US, unconditioned stimulus.

SLEEP, SLEEP STATES, AND SLEEP DEPRIVATION IN HUMANS AND IN RODENTS

The organization of the sleep-wake cycle differs in humans and rodents. First, laboratory rats and mice are nocturnal animals, and therefore spend the majority of daylight time resting, while nighttime is the active phase of the day. Second, rats are typically polyphasic, i.e., show repeated episodes of sleep during a 12:12 h light/dark (L/D) cycle. However, they spend around 70–80% of the night in wakefulness, and 70–80% of daytime asleep (Timo-Iaria et al., 1970). The daily amount and circadian distribution of sleep in mice (which are more active than rats in standard laboratory conditions) are similar to those of rats, though they exhibit variability across strains (Mistlberger, 2005).

Sleep in mammals includes two states: rapid eye movement (REM) sleep and non-REM (NREM) sleep. These two types of sleep are defined by electrophysiological signs detected in humans by a combination of electroencephalography (EEG), electrooculography and electromyography. NREM sleep, which in humans is divided into four stages of increasing depth, is characterized by high-voltage, low-frequency ("synchronized") wave activity and behavioral quiescence, with reduced activity in postural muscles. Besides the characteristic ocular movements, REM sleep (also known as paradoxical sleep) is defined by a low-voltage and high-frequency EEG pattern similar to that of wakefulness but, at variance with wakefulness, with suppressed vigilance and postural muscle tone. The loss of postural muscle tone during REM sleep is exploited in experimental SD procedures (see section Rem-Selective Sleep Deprivation).

NREM and REM sleep alternate in cycles, with NREM sleep preceding REM sleep epochs; NREM-REM sleep cycles have a fairly constant period, with a duration of 90 min in humans (Pace-Schott and Hobson, 2002). NREM-REM sleep cycles vary in duration as a function of the size of the animal and last about 12 min in the rat (McCarley, 2007).

All SD paradigms consist of partial or near-complete removal of sleep in an organism. Due to homeostatic regulation of sleep, SD causes a progressively accumulating sleep debt that results in increasingly greater efforts to maintain wakefulness over time.

With the exception of drug-induced insomnia, experimental SD in humans is invariably obtained by engaging subjects in a variety of activities that help maintain sufficient vigilance levels in order to avoid sleep. A crucial contribution in keeping subjects awake is, of course, motivation, whether provided by explicit rewards or by the knowledge of participating in a scientifically important activity. Furthermore, volunteer subjects know in advance about the duration of the experiment and are aware of the benign outcome of the experience.

These apparently self-evident considerations are very relevant when comparing experimental SD in humans and laboratory animals. By definition, human experimental SD is a "gentle," non-threatening procedure, whereas SD protocols in animals are inevitably contaminated by a degree of stress that may even represent an important confound for the interpretation of results. Indeed, as it will be outlined below, much effort has been put into developing SD protocols with the least amount of stress and other aversive conditions.

Twenty-nine distinct methods to induce insomnia in rats and mice have been reviewed by Revel et al. (2009). Examples are stress-related models, such as environmental changes (e.g., cage exchange, introduction of aversive odors) or discomfort (e.g., exposure to cold temperatures or pain); pharmacological models (e.g., administration of caffeine, psychostimulants, modafinil, etc.); genetic models (e.g., DBA/2J mice, clock gene mutants, orexin/hypocretin overexpression, etc.). Most of these protocols (Revel et al., 2009) create an underlying condition that is incompatible with sleep (hence the term insomnia). The experimental SD techniques in the focus of the present review aim instead at preventing an otherwise sleepy animal from falling asleep.

An important distinction needs to be made between *total* and *partial* or *state-dependent* SD. In the former procedure, all sleep is prevented for the desired amount of time, independent of sleep state. In partial SD, on the other hand, a specific sleep state is selectively targeted for deprivation. In the vast majority of cases, partial SD is restricted to REM sleep, although selective NREM SD can also be accomplished. Sleep state selectivity, total duration and temporal pattern of SD are chosen on the basis of the particular function under study or the particular deficit to be elicited.

It is commonly accepted that, even when carefully implemented, SD protocols only approximate their nominal goals. For example, SD can never be truly "total" for extended periods of time, as episodes of "microsleep" (short episodes of intrusion of sleep into wakefulness lasting as little as a few seconds) become inevitable with the accumulation of sleep debt (Friedman et al., 1979; Durmer and Dinges, 2005). Also, selective deprivation of one sleep state inevitably affects the amount and distribution of the other sleep state.

Two additional manipulations of the temporal schedule of SD, namely sleep restriction and sleep fragmentation are worth mentioning. With sleep restriction, the amount of sleep can be reduced by a predefined amount thus mimicking the human condition of abnormally late "bedtime" and/or early "wakeup" hours over an extended period of time (Leemburg et al., 2010). In sleep fragmentation procedures, sleep can be prevented for repeated but brief epochs, both within a single resting period and across several days. For example, animals can be forced to walk on a treadmill for 30 s, and be allowed to rest for 90 s over an extended period of time [see for example (Tartar et al., 2006)]. The obtained repeated arousals, i.e., brief, transient increases in EEG frequency, resemble conditions frequently observed in humans, such as those associated with either extrinsic stimulation (e.g., noise) or intrinsic events (e.g., sleep apnea) (Bonnet, 2005).

SLEEP DEPRIVATION PROTOCOLS IN RODENTS

TOTAL SLEEP DEPRIVATION BY GENTLE HANDLING

The "gentle handling" (GH) procedure is based on a direct interaction with the experimenter, who actively keeps the animal awake, and is by far the most popular method of total SD in the rodent. The procedure aims at minimizing the spurious effects of stress and of forced locomotor activity imposed by other methods (see below). GH requires the constant physical presence of dedicated and fully trained experimenters with whom animals must

be familiarized prior to the experiments. Animals, usually kept in their home cage during the SD procedure, are actively monitored by the experimenter, with or without the support of EEG and electromyographic recordings. The operator's task is to stimulate the animals just enough to keep them awake, whenever drowsiness or attempts to engage in a sleeping posture are observed, and/or if EEG signs of low-frequency activity appear. Two broad categories of stimulation can be distinguished: (1) passive exposure to external stimuli, (2) engagement in spontaneous exploratory and locomotor behavior.

(1) In the case of passive exposure to external stimuli, the animal can be subjected to external stimulation, such as mild noises, tapping or gentle shaking of the cage, or by direct contact with the animal either through a soft brush or by hand. A certain degree of induced stress should always be taken into account as SD manipulations are often associated with elevated corticosterone levels (Longordo et al., 2009). Repeated handling *per se* causes alterations in locomotor activity and an increase in circulating corticosterone levels in comparison with undisturbed animals (Meerlo et al., 2008; Longordo et al., 2011). When comparing "mild" stimulation, such as tapping on the cage, to "stressful" direct handling of the animals at 30 min intervals, it was found that corticosterone levels in the former group were comparable to those of control animals, but were three times as high in handled animals (Kopp et al., 2006). In SD by GH studies, control experiments should be aimed at teasing out the effects of stress and of generic sensorimotor stimulation from the specific consequences of sleep loss. For example, SD by GH administered during the first 6 h of the resting circadian phase immediately after the acquisition of a fear conditioning paradigm affected the consolidation of fear memory, while an equivalent period of SD carried out during the active phase did not (Hagewoud et al., 2010). This result supports the hypothesis of a specific role of sleep loss on memory consolidation, rather than a non-specific effect of the handling procedures (but see Cai et al., 2009).
(2) Protocols aimed at engaging exploratory activity may include the introduction of novel objects or nesting material in the cage (Tobler et al., 1997; van der Borght et al., 2006), which typically leads to active exploratory behavior and has been shown to help maintain wakefulness (Hairston et al., 2005). Stress caused by stimuli that elicit spontaneous exploratory behavior, reflected by corticosterone levels, is indistinguishable from that of controls, which would make the latter approach preferable, in principle (Kopp et al., 2006). On the other hand, stimulating the animal by enriching the environment in order to enforce wakefulness may in some cases represent a confound for subsequent behavioral and cognitive tests. For example, performance in the novel object recognition task (NOR, see below) may be altered by previous exposure to unfamiliar objects in the cage.

The choice of protocol and the intensity and frequency of stimulations are directly proportional to SD duration. In our experience, rats and mice are easily kept awake by mild stimulation for the first few hours, but direct handling becomes necessary beyond 5–6 h of SD. Adapting the amount of stimulation to each animal's instantaneous vigilance inevitably introduces variability among different animals but, on the other hand, presumably minimizes stress.

An experienced operator can administer GH to a small number of rodents at once (in our own lab, 6 would be considered the maximum), given that detection of sleep-seeking behavior and/or of sleep-related brain activity would be less effective as the number of monitored animals increases. When properly executed, SD by GH effectively suppresses nearly all sleep activity in rats and mice. In rats, SD by GH can reduce NREM sleep by 92% and REM sleep by 100% (Franken et al., 1991), even though the occurrence of microsleep needs to be taken into account. On the other hand, the method is labor-intensive and may be difficult to fully standardize across laboratories and even between operators within the same lab.

AUTOMATED TOTAL SLEEP DEPRIVATION

Total SD can be administered by forcing specific patterns of locomotor activity, either continuously or as soon as the animal shows behavioral and/or electrophysiological signs of impending sleep. Several automated devices have been devised to make sleep impossible in rodents.

Continuously moving treadmills or rotating wheels are commonly used apparatuses to achieve total SD in animals (Borbely and Neuhaus, 1979). Another automatic method to enforce total SD is the "alternating platform." The apparatus is made of two small platforms, placed in a water tank, which continuously and alternatively emerge from and are submerged by water, thus forcing the rat or mouse to a permanent motion in order to avoid contact with water. The method has been reported to completely abolish sleep (Pierard et al., 2007).

The obvious advantage of such procedures is that the quality and amount of stimulation can be standardized and made equal for all experimental animals. On the other hand, the concern that effects measured after the procedure may be a consequence of stress and fatigue rather than of sleep loss *per se* is higher in these paradigms than in GH.

In an attempt to control for the contribution of the SD-enforcing stimulation to results, Rechtschaffen and coworkers (Rechtschaffen et al., 1983; Bergmann et al., 1989; Rechtschaffen and Bergmann, 1995) introduced the "disk-over-water" (DOW) apparatus, which to this day is regarded as the reference automated technique for total SD. In this paradigm, one experimental and one control rat (or mouse) are housed on each side of a cage split in half by a divider, and with the floor replaced by a rotating disk suspended over a tank of shallow water. As soon as the experimental rat (or mouse) enters a "forbidden" vigilance stage, the disk starts rotating, forcing the animal to walk in the opposite direction to avoid falling into the water. The control animal is able to sleep whenever the experimental one is spontaneously awake and therefore the disk is not rotating. Thus, both the experimental and control animals receive the same mild sensory stimulation and a similar locomotor load. Inevitably, if both animals attempt to sleep at the same time, they are both woken up by the rotating disk. It is therefore unavoidable that controls are at least partially

sleep deprived as well. In one typical total SD study (Everson et al., 1989), total sleep time was reduced by 91% in experimental animals, and by 28% in yoked controls, compared to baseline recordings.

It should be noted that the DOW method has typically been administered over extended periods of time (at least several days, and up to 1 month) to animals kept in constant light conditions. The L/L (light/light) condition rapidly disintegrates the diurnal sleep/wake cycle (Eastman and Rechtschaffen, 1983), thereby making sleep pressure homogeneous in both SD and control animals, independent of subjective circadian rhythmicity. However, while this manipulation allows a direct comparison between experimental groups, the disruption of the circadian rhythm adds complexity to the interpretation of any subsequent cognitive deficits. In the context of studies of cognitive functions, one could argue that for a relatively brief period of SD in the DOW apparatus the two animals should be kept at opposite light-dark phases. To our knowledge, there are no reports on the use of the DOW method for the relatively short SD periods (<24 h) typically adopted in cognition studies.

Another method of total SD in rodents is represented by the "grid over water" (Shinomiya et al., 2003). Placing a rat on a grid floor suspended over a tank filled with water significantly reduces total NREM and REM sleep time and increases sleep latency and wakefulness total time compared to rats placed on sawdust. This method has been proposed for the evaluation of the hypnotic properties of drugs (Shinomiya et al., 2003).

REM-SELECTIVE SLEEP DEPRIVATION

The striking differences in brain activity between sleep states have long represented a major focus for sleep research, and methods that allow selective deprivation of each sleep state (state-selective sleep deprivation, SS SD) have therefore received much attention.

In humans, SS SD performed with the aid of polygraphic monitoring allows selective deprivation of either REM sleep or deep NREM sleep (usually stages 3–4). Deprivation limited to superficial NREM sleep is impossible to accomplish in practice, as subjects would have to be woken up immediately upon falling asleep, which would be equivalent to total SD. While polygraphic monitoring has also been used in rodents, the sudden drop in muscle tone at the onset of REM sleep makes it possible to implement REM-selective SD in the rodent in the absence of electrophysiological recordings.

REM-selective SD can be easily obtained in rodents by means of the so-called "flowerpot method". Initially employed to study sleep in cats (Jouvet et al., 1964), the procedure has since been adapted to deprive mice and rats of REM sleep. In the original version, the animal is placed in a water tank, on top of a small platform (traditionally an upside-down clay flower pot) slightly raised above the water surface (Morden et al., 1967; Mendelson et al., 1974). After some preliminary adaptation to the apparatus, the animal is left undisturbed in the water tank for the duration of the experiment. The platform allows the animal to crouch and to obtain NREM sleep *ad libitum*. However, at the onset of each REM period, the loss of muscle tone causes the neck to relax and the snout to touch the water, thus arousing the animal. As the duration of the SD period increases, the animal increasingly loses balance and falls in the water. The typical control is represented by an animal placed in a similar environment but on a platform sufficiently large to allow for a fully relaxed posture and hence REM sleep.

Because of its simplicity, the technique has been widely used, contributing to an understanding of the roles and mechanisms of REM sleep. It should be pointed out, however, that although the procedure primarily targets REM sleep, a substantial loss of NREM sleep has been also reported (Grahnstedt and Ursin, 1985; Machado et al., 2004). Furthermore, the procedure is accompanied by a non-negligible amount of stress (Revel et al., 2009) which may confound the interpretation of the results. In order to reduce stress, the protocol has been modified by placing multiple platforms in a larger tank, and by sleep-depriving several animals at once (van Hulzen and Coenen, 1981). Thus, animals are free to move around the cage, jumping from platform to platform, and to interact with their cohorts, thereby reducing the stress caused by immobility and social isolation (but see Suchecki et al., 1998; Machado et al., 2004).

As mentioned above, the flowerpot method allows to perform SS SD without the aid of electrophysiological recordings. Unfortunately, no comparable, straightforward technique for selective NREM SD is available. Where polygraphic recordings are available, SS SD in rodents can be implemented in a similar way as in humans. For example, upon entering a forbidden zone (either REM sleep or high-amplitude NREM), animals can be aroused in ways that are comparable to those used in GH, either by giving objects to play with or by introducing acoustic and tactile stimuli (Endo et al., 1997).

Another protocol introduced for REM SD is the "head-lifting method," proposed to reduce some of the disadvantages encountered with the majority of REM SD methods (Datta et al., 2004). In this procedure, the beginning of each REM sleep episode is identified by observation of ongoing polygraphic records. From the adjacent room, the experimenter presses a mechanical lever within a few seconds from REM sleep onset, so that the animal's head is gently lifted and the animal is woken up. Using this method, an experienced operator can terminate REM sleep episodes within 3–5 s of their onset.

The head-lifting method is thought to minimize extraneous stress and physical activity and eliminate the need for the experimenter's physical proximity to the rat. During a 6 h recording session, the head-lifting method has been reported to successfully eliminate 90–95% of total REM sleep with no significant reduction of SWS (Datta et al., 2004).

SLEEP DEPRIVATION-INDUCED MEMORY IMPAIRMENT IN RODENTS

As mentioned above, much effort has been devoted in humans to the study of the impact of SD on various cognitive domains, including vigilance and basic attentional processes, memory, as well as more complex real-world tasks.

While it is clear that all mammals share a fundamental physiological need for sleep and that prolonged SD has a dramatic impact on the organism, some important consequences of SD on high-level human cognition may well be beyond the reach of animal models. As with any other field of comparative study,

reproducing in rodents the SD-induced deficits observed in humans is made difficult by the intrinsic differences between the species sleep-wake cycles. Thus, utmost care should be devoted to the choice of the behavioral/cognitive task and interpretation of the results to obtain meaningful insights from SD studies.

In rodents, investigations on the role of sleep loss and SD have focused on their effects on learning and memory performance (**Tables 1**, **2**). Several examples are presented in the following paragraphs.

SPATIAL REFERENCE MEMORY IN THE MORRIS WATER MAZE

The study of spatial memory in rodents has focused on the innate ability to find and remember locations using any available, distant (allothetic) cues. This type of learning requires the formation of a spatial map of the environment. The most commonly adopted task to test spatial reference memory is the "Morris water maze" (MWM) (Morris et al., 1982; Morris, 1984). The basic setup consists of a circular tank filled with water rendered opaque by diluted inert paint. Animals are placed in the water and swim until they stumble upon and climb on a slightly submerged, and therefore invisible platform. On subsequent trials, animals learn to find the platform, always placed in the same position, more and more efficiently, aided by the visual cues available in the room. Training sessions consist of at least 4 trials (with each trial starting at a different location along the perimeter of the pool) and are typically repeated over a period of a few days. Performance can be assessed during training by measuring the distance covered and the time elapsed searching the escape platform. Importantly, in a final probe trial, the platform is removed from its usual location, and the time spent by the animal searching for the platform at the expected location is interpreted as a measure of *spatial, hippocampus-dependent memory* (Morris, 2001). In order to dissociate hippocampus-independent behaviors, the platform can be made visible in control trials, thereby allowing to control for sensory and motor abilities, visual acuity, motivation, and thigmotaxis (Packard and McGaugh, 1992).

Deficits in the ability to memorize the submerged platform's location have been reported as a consequence of both REM-selective SD and total SD by GH. The first study (Smith and Rose, 1996) was designed to establish the most appropriate time window following the daily training session for the administration of REM SD in rats. The most severe learning deficits were obtained when 4 h REM SD by the flowerpot method were administered beginning 5 h after the end of training. Interestingly, a recent report has documented a lack of substantial effects of 6 h REM SD preceding training in the MWM (Walsh et al., 2011).

A continuous 72 h REM SD with the flowerpot method administered to rats during their training period was shown to impair both the acquisition rate in the MWM and the ability to remember the position of the platform in the subsequent probe test (Zhao et al., 2010). Interestingly, the detrimental effects of pre-training REM SD on spatial learning and memory in the MWM are more severe in female than in male rats (Hajali et al., 2012).

In a study of SD-dependent alterations in molecular mechanisms of synaptic plasticity (Guan et al., 2004), rats were given 6 h of total SD by means of GH, starting at *lights-on* time (which effectively makes the length of the sleepless epoch longer when compounding the previous, normally active, wakefulness period). SD was immediately followed by a 12-trial training session in the MWM. The loss of sleep did not cause significant impairments in the animals' learning rate compared to controls. However, a probe trial without the platform performed 24 h later showed in the sleep-deprived animals a lack of preference for the expected platform location, suggesting an impairment of spatial reference memory despite improved performances over time (Guan et al., 2004).

Rats subjected to a 5-day period of total SD by the DOW method and trained in the MWM immediately afterwards were found to be impaired in spatial learning and even more severely impaired in a test of long-term spatial memory performed 12 h later (Chang et al., 2009). Brief epochs of total SD repeated over an extended period of time also cause deficits in the MWM. At the end of a 30-day period during which mice were sleep-deprived every day for 3 h by means of GH, the animals received 3 daily sessions of training and a test (probe) session 24 h afterwards, and showed significantly impaired spatial learning and spatial memory retention (Xu et al., 2010). Similarly, decrements in the acquisition of the platform location in the MWM have been

Table 1 | Synopsis of impairments in memory function after sleep deprivation in laboratory rats and mice.

Memory function	Test	TSD			REM-SD			SR		SF
		≥6 h	10–12 h	>1d	4–6 h	12–24 h	>72 h	TSR*	REM-SR*	
Spatial memory acquisition	MWM			r, m	r	r	r	r, m		r
Spatial memory retention	RAM		r		r					
	MWM			r, m	r	r	r	r, m		r
Avoidance acquisition	RAM				r					
	Fear conditioning, avoidance test				r, m	r, m	r, m	r		
Contextual memory	Fear conditioning, avoidance test	m	m		r, m	r, m	r, m		r	
Working memory	Y-maze, RAM	r	r, m							m
Object recognition memory	NOR	r, m								m

Brief and repeated sleep restriction episodes.

Abbreviations: m, r, tested in mice, rats, respectively; MWM, Morris water maze; NOR, novel object recognition; RAM, radial-arm maze; REM-SD, REM-selective sleep deprivation; REM-SR, REM-selective sleep restriction; SF, sleep fragmentation; SR, sleep restriction; TSD, total sleep deprivation; TSR, total sleep restriction.

Table 2 | Studies on sleep deprivation-induced memory impairment in laboratory rats and mice (1981–2012).

PMID	References	Species	Strain	SD method	SD duration	SD time schedule	SD vs. training	Test	EEG monitoring
22989412	Inostroza et al., 2013	r	LE	GH	80 min	Continuous	After	NOR	n
23238166	McCoy et al., 2013	r	LE	Activity wheel	18 h	Repeated for 5 days	After	MWM	n
23082139	Kumar and Jha, 2012	r	W	GH, rotating disk	6 h	Continuous	After	FC	y
22654204	Yang et al., 2012	r	SD	GH	4 h	Repeated for 7 days	After	MWM	y
22521334	Fernandes-Santos et al., 2012	m	Swiss	GH	6 h	Continuous	After	PM-DAT, FC, PA	n
22321457	Leenaars et al., 2012	r	W	Rotating wheels	3–12 h	Continuous	After	OT	n
22192378	Hajali et al., 2012	r	W	Flower pot	72 h	Continuous	Before	MWM	n
21677190	Walsh et al., 2011	r	SD	Flower pot	6 h	Continuous	After	MWM	y
21788501	Rolls et al., 2011	m	C57BL	Electric stimulation	30 s	Repeated for 4 h	After	NOR	y
21624432	Aleisa et al., 2011	r	W	Flower pot	24 h	Continuous	After	RAWM	n
21356250	Esumi et al., 2011	r	W	Flower pot	96 h	Continuous	Before	AT	n
21295147	Chowdhury et al., 2011	r	W	GH	6 h	Continuous	After	OT	n
21120129	Patti et al., 2010	m	EPM-M1	GH[a]; flower pot[b]	[a]6; [b]72 h	Continuous	Before, after	PM-DAT	n
20717746	Xu et al., 2010	m	C57BL	GH	3 h	Repeated for 20 days	Before	MWM	n
20614860	Moreira et al., 2010	r	W	Flower pot	96 h	Continuous	Before	AT	n
20561181	Legault et al., 2010	r	SpD	Flower pot	4 h	Continuous	After	RAM	n
20495497	Zhao et al., 2010	r	SpD	Flower pot	72h	Continuous	Before	MWM	n
20394312	Alhaider et al., 2010	r	W	Flower pot	24 h	Continuous	Before	RAWM	n
20050994	Hagewoud et al., 2009	m	C57BL	GH	6–12 h	Continuous	Before	NART	n
19962429	Dubiela et al., 2009	r	W	Flower pot	96 h	Continuous	Before	PA	n
19850085	Ramanathan et al., 2010	r	SpD	GH	6 h	Continuous	Before	Ym	n
19847264	Vecsey et al., 2009	m	C57BL	GH	5 h	Continuous	After	FC	n
19794184	Cai et al., 2009	m	C57BL	GH	12 h	Continuous	After	FC	
19645967	Ward et al., 2009	r	FN	Treadmill	24 h	Continuous	Before	MWM	n
19643093	Ward et al., 2009	r	FN	Treadmill	24 h	Continuous	Before, after	MWM	n
19627456	Chang et al., 2009	r	W	DOW	5 d	Continuous	Before	MWM	n
19619610	Tian et al., 2009	r	SpD	Flower pot	6 h	Continuous	After	FC	n
19597374	Wang et al., 2009	r	SpD	Flower pot	72 h	Continuous	After	MWM	n
19542091	Li et al., 2009	r	SpD	Flower pot	48 h	Continuous	After	MWM	n
19444749	Palchykova et al., 2009	m	OF1	GH	6 h	Continuous	After	NOR	n
19193874	Bjorness et al., 2009	m	KOm	Treadmill	48 h	Continuous	During	RAM, PA	y
19186164	Halassa et al., 2009	m	KOm	GH	6 h	Continuous	After	NOR, FC	y
19014078	Novati et al., 2008	r	W	Rotating wheels	20 h	Repeated for 8 days	Before	FC	n
18985181	Kalonia et al., 2008	r	W	Grid over water	72 h	Continuous	Before	Active avoidance, MWM	y
18775445	Ruskin and Lahoste, 2008	m	C57BL	Flower pot	24 h	Continuous	Before	FC	n
18707010	Alvarenga et al., 2008	r	W	Flower pot	96 h	Continuous	Before, after	PM-DAT	n
18674519	Yang et al., 2008	r	SpD	Flower pot	120 h	Continuous	After	MWM	n

(Continued)

Table 2 | Continued

PMID	References	Species	Strain	SD method	SD duration	SD time schedule	SD vs. training	Test	EEG monitoring
18329112	Perry et al., 2008	r	W	Flower pot	96 h[a] and 18 h[b]	[a]Continuous and [b]repeated for 21 days	Before	AT	n
18281713	Calzavara et al., 2009	r	SHR	Flower pot	96 h	Continuous	Before	FC	n
17920644	Silvestri and Root, 2008	r	SpD	Flower pot	6 h	Continuous	After	FC	n
17698177	Pierard et al., 2007	m	C57BL	Automated TSD	10 h	Continuous	Before	SA-WM	y
17157993	Fu et al., 2007	r	SpD	Flower pot	6 h	Continuous	After	FC	n
16876303	Silva et al., 2007	m	M	Flower pot	24 h	Continuous	Before	PA	n
16817877	Tartar et al., 2006	r	SpD	Treadmill	24–72 h	Continuous	Before	MWM	y
16423541	Palchykova et al., 2006	m	OF1	GH	6 h	Continuous	After	NOR	n
16376302	Chen et al., 2006	m	C57BL	Flower pot	24 h	Continuous	Before	FC	n
16325867	Ruskin et al., 2006	r	SpD	Flower pot	72 h	Continuous	After	MWM	n
16014798	Hairston et al., 2005	r	R	GH	6 h	Repeated for 5 days	After	MWM	y
15777764	Dubiela et al., 2005	r	W	Flower pot	96 h	Continuous	Before	AT	n
15763570	Silvestri, 2005	r	SpD	Flower pot	6 h	Continuous	After	FC	n
15582679	Legault et al., 2004	r	SpD	Flower pot	4/10 d	Repeated for 10 days	During	RAM	n
15582021	de Oliveira et al., 2004	r	W	Flower pot	72 h	Continuous	before	AT	n
15341794	Silva et al., 2004a,b	m	EPM-M1	Flower pot	72 h	Continuous	Before, after	AT	n
15317872	Su et al., 2004	r	SpD	Flower pot	12–24 h	Continuous	After	FC	n
15262203	Guan et al., 2004	r	SpD	GH	6 h	Continuous	Before	MWM	n
15182321	Ruskin et al., 2004	r	SpD	Flower pot	72 h	Continuous	Before	FC	n
15033349	Silva et al., 2004a,b	m	EPM-M1	Flower pot	72 h	Continuous	Before	AT	y
14960614	Datta et al., 2004	r	SpD	Head-lifting	6 h	Continuous	After	AT	y
14573548	McDermott et al., 2003	r	SpD	Flower pot	72 h	Continuous	Before	FC	n
12954399	Wang et al., 2003	r	W	Flower pot	24 h	Continuous	After	AT	n
12788510	Moreira et al., 2003	r	W	flower pot	96 h	Continuous	Before	AT	n
12773581	Graves et al., 2003	m	C57BL	GH	5 h	Continuous	After	FC	n
12644281	Smith and Kennedy, 2003	r	SpD	Flower pot	48 h	Continuous	After	AT	n
11809508	Dametto et al., 2002	r	W	Flower pot	96 h	Continuous	Before	AT	n
11527327	Le Marec et al., 2001	r	SpD	Flower pot	4 h	Continuous	Before	MWM	n
10958153	Bueno et al., 2000	r	W	Flower pot	96 h	Continuous	Before	AT	n
10866356	Kennedy et al., 2000	r	SpD	Flower pot	24–48–96 h	Continuous	After	AT	n
10857657	Beaulieu and Godbout, 2000	r	SpD	Flower pot	12 h	Continuous	Before	MWM	n
10837820	Prathiba et al., 2000	r	W	Flower pot	96 h	Continuous	Before	AT	n
10604833	Youngblood et al., 1999	r	W	Flower pot	96 h	Continuous	During	MWM	n
9619997	Smith et al., 1998	r	SpD	Flower pot	4–12 h	Continuous	After	RAM	n
9438789	Smith and Rose, 1997	r	SpD	Flower pot	4 h	Continuous	After	MWM	n
9035255	Youngblood et al., 1997	r	SpD	Flower pot	96 h	Continuous	During	MWM	n
8848497	Smith and Rose, 1996	r	SpD	Flower pot	4 h	Continuous	After	MWM	n

(Continued)

Table 2 | Continued

PMID	References	Species	Strain	SD method	SD duration	SD time schedule	SD vs. training	Test	EEG monitoring
8788869	Gruart-Masso et al., 1995	r	W	Flower pot	5 h	Continuous	Before	AT	n
7800747	Bueno et al., 1994	r	W	Flower pot	24–72–96 h	Continuous	Before	AT e FC	n
1896504	Coll-Andreu et al., 1991	r	W	Flower pot	3–6 h	Repeated for 5 days	After	AT	n
3413257	Marti-Nicolovius et al., 1988	r	W	Flower pot	5 h	Repeated for 5 days	After	AT	n
3212058	Smith and Kelly, 1988	r	SpD	Flower pot	24–72 h	Continuous	After	AT	n
3174846	Ambrosini et al., 1988	r	W	Flower pot	3 h	Continuous	After	AT	
7163344	Harris et al., 1982	r	W	Flower pot	72 h	Continuous	Before	AT	n
6891076	van Hulzen and Coenen, 1982	r	W	Flower pot	72 h	Continuous	Before	AT	n
7178252	Smith and Butler, 1982	r	SpD	Flower pot	4 h	Continuous	After	AT	n
7053243	Kitahama et al., 1981	m	C57BR and C57BL	Flower pot	10 or 24 h	Continuous	After	AT	n

Abbreviations: AT, avoidance test; FC, fear conditioning; FN, Fisher Norway rats; GH, gentle handling; KOm, knock-out mice; LE, long evans rats; m, mice; MWM, Morris water maze; NOR, novel object recognition; OF1, Oncins France 1 mice; OT, operant task; PA, passive avoidance; r, rats; PM-DAT, plus-maze discriminative avoidance task; RAM, radial arm maze; RAWM, radial arm water maze; SD, sleep deprivation; SpD, Sprague Dawley rats; SHR, spontaneously hypertensive rats; W, Wistar rats.

reported as a consequence of 6 h SD which occurred repeatedly during a 4-day training period (Hairston et al., 2005).

The so-called "place learning set task," a modified MWM task, is regarded as a rodent equivalent of human short-term memory tests such as the digit-span task (Whishaw, 1985). The procedure consists of pairs of identical trials, separated by a short delay (up to 1 min). Trial pairs are separated by a substantially longer delay (5–30 min) and the starting position of the platform changes from one trial pair to the next. Each session consists of a variable number of trial pairs (8–12) and the entire training consists of several daily sessions, with the platform location changing every day. This version of the MWM allows to probe distinct skills and memory types. Differences in escape latency between the two trials in each pair are taken as a measure of either short-term memory (Whishaw, 1985; Ruskin et al., 2006) or working memory (Youngblood et al., 1997, 1999; Yang et al., 2008), whereas performance delta between trial pair sets is ascribed to spatial reference memory. Overall performance gains across training sessions reflect the ongoing learning process of the general procedure, independent of the specific platform location (Youngblood et al., 1997).

A decrease in learning rate was observed in this modified version of the MWM after 72 h of REM SD administered before testing. The deficit has been interpreted as due to either poor spatial working memory or to slow learning of the task rules (Ruskin et al., 2006). With the same paradigm, severe impairment of spatial reference memory performance with no deficit of spatial working memory has been reported in animals repeatedly tested over a 4–5-day REM SD period (Youngblood et al., 1997, 1999; Yang et al., 2008).

Similarly, sleep fragmentation for 24 h obtained with the treadmill method caused deficits in the retention of spatial reference memory (tested 24 h after training) in spite of a normal learning curve, suggesting selective interference of prior sleep disturbances with the consolidation of spatial memories (Ward et al., 2009).

In yet another variant of the MWM, the so-called "delayed alternation navigation task," animals are placed in the water always in the same spot, and the platform is hidden at one of two locations, alternating on each trial. A short REM SD (from 4 to 8 h) preceding the session was found to disrupt the animals' performance with no deficits in the conventional (allocentric) version of the task, suggesting that hippocampus-independent, frontal-like performance could be more susceptible to a short period of REM SD than hippocampus-dependent spatial memory (Beaulieu and Godbout, 2000; Le Marec et al., 2001).

Dissociations in the effects of total SD on spatial *vs.* non-spatial memory have recently been investigated with a novel task (Pierard et al., 2011). The apparatus consists of a square open field whose floor can be changed both in color and texture, and with a hole placed in each of its 4 corners. In the spatial-only version of the task, the animal learns to find food pellets hidden in one of two diagonally opposite holes, solely aided by visual cues external to the open field (allocentric). The floor remains unchanged and neutral with respect to the outcome of these trials. After a retention interval followed by an epoch of total SD, spatial memory is tested by placing animals in the same open field, with all 4 holes unbaited, and counting the number of visits to the previously

rewarded holes. In the context-dependent version of the test, each of the 2 baited holes is associated with a floor of specific color and texture. During the test phase, only one floor is presented, and the number of visits to the associated hole is taken as a measure of context-dependent memory. Total SD for 10 h caused a deficit in the more complex contextual task, but not in spatial memory *per se* (Pierard et al., 2011).

SPATIAL WORKING MEMORY AND THE RADIAL ARM MAZE

Both in humans and animals, working memory can be defined as the ability to store and manipulate the information necessary to accomplish cognitive tasks such as learning within one session, but not between different sessions (Baddeley, 1992; Dudchenko, 2004). This function can thus be distinguished from long-term memory because of its "transient" character. Spatial working memory, that is working memory for locations, has been assessed in rodents with a variety of tests. For example, the "spontaneous alternation task" and the "novel arm recognition task" exploit the animals' innate exploratory behavior, which leads to spontaneously alternate visits between arms in a maze on each trial, or to spend more time in the novel than in previously explored arms, without the use of behavioral reinforcers. Retaining the necessary information in order to successfully alternate between arms as well as spending more time in the novel arm compared to the previously explored arms is assumed to require intact working memory. While spontaneous alternation paradigms are considered more dependent on the frontal cortex (Verma and Moghaddam, 1996), spatial memory guided by external cues is highly dependent on the hippocampal formation (Winocur and Moscovitch, 1999; Yoon et al., 2008; Alhaider et al., 2010). Spatial working memory in rodents has been challenged with 6–10 h of total SD. In these studies, animals were habituated to the apparatus and then subjected to total SD before being tested in the maze. Total SD was shown to impair the animals' alternation rate as well as the time spent in the novel arm relatively to the previously explored arms (Pierard et al., 2007; Hagewoud et al., 2010).

A widely used procedure to assess both spatial working memory and spatial reference memory in rodents is the "radial arm maze" (RAM) (Olton and Samuelson, 1976). At odds with the previously described tests, in the RAM reward is adopted to motivate behavior. The maze consists of a central platform and eight arms originating from it. Several prominent extramaze visual cues are usually situated around the testing room. In the original procedure, animals are trained to find food pellets placed at the end of each arm. Since only one pellet per arm is available, the optimal strategy is to visit each of the eight arms only once, which requires to hold in memory which of the arms have already been visited (Olton and Samuelson, 1976).

Dissociations between spatial reference and spatial working memory can be investigated with the RAM using a paradigm in which four arms are baited while the other four never contain food (Legault et al., 2004). After a number of training sessions, visits to the unbaited arms are regarded as long-term reference memory errors, while repeated visits to previously baited arms are scored as working memory errors (see also Olton and Papas, 1979). REM-selective SD administered during the 4 h period immediately following a training session in the RAM was found to elicit a deficit in spatial reference memory but not in spatial working memory (Smith et al., 1998; Legault et al., 2004).

An alternative version of the RAM is the "win-shift paradigm," which has also been used to assess hippocampus-dependent spatial memory. During daily training, animals are placed at the center of the maze and all arms are opened after a brief delay. When an animal enters a given arm, the doors to the other arms are closed. When the animal returns to the central platform, its door is also closed; after a delay, all eight doors are again opened and the animal can choose another arm. The routine is repeated until all baits have been obtained or until a maximum trial duration (10 min) is reached. The time taken by an animal to complete the RAM task each day and the number of baits eaten by an animal during a trial could be used as indicators of rate of learning. Learning rate in the win-shift paradigm is severely impaired when a period of 4 h REM SD occurred immediately after each daily session of training (Legault et al., 2010).

A hybrid of the RAM and the MWM, the "radial arm water maze" (RAWM) (Diamond et al., 1999; Alzoubi et al., 2009; Alhaider et al., 2010), has been adopted to explore spatial memory and the integrity of related hippocampal function after SD. A four- or six-arm maze is placed inside a circular water tank, with submerged walls almost reaching the surface, and an escape platform located at the end of one of the arms (goal arm). The procedure differs from a canonical MWM in that animals entering the wrong arm are forced to swim back to the central area and then swim into another arm until they eventually find the goal arm. In each trial of this task, the animal is placed in the water at the end of a non-goal arm and is allowed a predefined amount of time to reach the goal platform. Rats submitted to 12 h REM SD commit significantly more errors than controls in finding the hidden platform both during the acquisition phase of the task and during the short-term memory test administered 30 min after the end of the learning phase (Alhaider et al., 2010). Moreover, rats that successfully learn to find a submerged platform fail to locate it if they are tested at the end of a 24 h REM SD administered immediately after the training (Aleisa et al., 2011).

CONTEXTUAL OR PAVLOVIAN FEAR CONDITIONING OR CUED LEARNING

The effects of SD on associative learning, i.e. the ability to form new or to modulate existing associations, have been extensively explored. One of the most commonly adopted paradigms is classical "fear conditioning" (FC). In the "cued" version of the paradigm, the animal is trained to learn the association between an initially neutral conditioned stimulus (CS, usually an auditory tone), and a biologically relevant unconditioned stimulus (US, usually a mild electric footshock). After a single pairing between the cue and the shock, the CS will predict the occurrence of the US and elicit a response similar to that caused by the US. In the "uncued" task, the association is established between the learning context and the US. Fear conditioning is considered a rodent model of declarative memory (Anagnostaras et al., 2001), and both contextual and cued learning are amygdala-dependent, whereas contextual learning is also hippocampus-dependent (Ehrlich et al., 2009; reviewed by Radulovic and Tronson, 2010). SD has been shown to interfere

with the consolidation and acquisition of contextual FC but not of cued FC.

Selective REM SD preceding training has been repeatedly reported to impair contextual FC in rats (Ruskin et al., 2004; Tiba et al., 2008). In particular, control animals show a pronounced freezing response when placed in the same environment where they previously experienced electric shocks, whereas sleep-deprived animals fail to show such behavior. Similar negative effects on contextual fear memory have been reported after a chronic sleep restriction (rats kept awake for 20 h and allowed to sleep for 4 h over 3 consecutive days) preceding training (Ruskin and Lahoste, 2008).

Moreover, the critical time window for contextual FC has been identified as the 5 h immediately following 5 h of training. In fact, total SD by GH immediately after training has been shown to affect the consolidation of memory for contextual FC tested 24 h after training (Graves et al., 2003; Vecsey et al., 2009; Hagewoud et al., 2010). Interestingly, no impairment in memory for contextual fear has been found when the SD period was initiated 5 h after the end of training (Graves et al., 2003; but see Su et al., 2004).

An alternative FC protocol is the "inhibitory avoidance task," which has been widely used to study learning and memory in rodents. This task employs a 2-way shuttle-box with a guillotine door placed between the 2 chambers. One chamber is illuminated, while the other is in the dark. In the training session, the animals are individually placed in the illuminated chamber, facing away from the guillotine door. When the animal spontaneously enters the darkened chamber, a foot shock is applied through the grid floor, and the animal is moved back into the lit, safe chamber. The procedure is repeated until the animal learns about the association and does not cross the opening for 2 min. In the test sessions, the animals are again placed in the illuminated chamber and free to walk into the dark chamber. The latency to enter the dark chambers is interpreted as a function of recall of the aversive association, so shorter latencies may indicate cognitive impairment. Differently from the FC paradigm where the CS and US are delivered independently from the animal's behavior, in the avoidance task shock delivery is contingent on the animal's response and is therefore considered to test operant or instrumental learning.

In the inhibitory avoidance paradigm, REM SD preceding training does not prevent the animal from forming the association, as shown by normal rates of acquisition of the correct response. On the other hand, compared to cage controls, SD animals showed shorter latencies in entering the dark chamber during the test trials performed 24 h later (Moreira et al., 2003; Silva et al., 2004a,b; Esumi et al., 2011). This behavior could depend on impaired recall of the association at the time of testing or, more likely, on a disruption of the long-term storage of the environmental contingency.

Conflicting results have been reported on FC learning when REM SD is administered after training. In one study, 6 h of REM SD caused performance impairments in rats tested immediately after the deprivation procedure (Datta et al., 2004; Silva et al., 2004b). In another study, performance was normal immediately after 72 h of REM SD, but was severely affected when re-tested one week later (Silva et al., 2004a).

Periods of 9–12 h and 17–20 h after training have been identified as critical time windows for avoidance learning (Smith and Butler, 1982; Smith and Lapp, 1986). Increases of REM sleep after learning, which are considered to reflect a homeostatic response to the increased demands for memory consolidation (see Walker and Stickgold, 2004 for a review), are observed during these temporal intervals and REM SD is considered to be maximally effective in impairing response acquisition if administered during such times. It is worth mentioning that also paradoxical facilitatory effects on retention performance have been reported in an avoidance learning task when 24 h REM SD was adminstered immediately after training (Gisquet-Verrier and Smith, 1989).

Finally, the "elevated plus-maze (EPM) discriminative active avoidance" (or plus-maze discriminative avoidance task, PM-DAT) paradigm has been used in mice as alternative to classical protocols of associative learning. This task has the advantage of evaluating at the same time two closely related behavioral phenomena, memory and anxiety, as well as locomotor activity (Silva et al., 1997; Silva and Frussa-Filho, 2000). The EPM consists of four arms standing at some distance from the floor, two of which offer no protection while the other two are surrounded by opaque walls, and are typically preferred by rodents. The time spent in the closed *vs.* open arms is considered a measure of high-anxiety trait. In this version of the EPM, every time the animal enters a previously selected enclosed arm, aversive stimuli such as light and noises are presented and persist until the animal leaves the arm. At a variable delay after the conditioning, during the test session, animals are placed in the same apparatus but receive no aversive stimulation. Time spent in the aversive or non-aversive closed arms, time spent in the open arms as well as the total number of entries in any arms are measured. Decreases in the amount of time spent in the aversive arm during 10 min-training, measured minute by minute, is interpreted as learning of the task, whereas memory is measured as the percent time spent in the aversive *vs.* non-aversive enclosed arms in the test session. A significant difference between the time spent in the aversive and non-aversive enclosed arms in the test session is considered to reflect information retention (Silva et al., 1997). This paradigm allows also an assessment of anxiety levels by the percent of time spent in the open arms.

REM SD has been reported to produce different effects on acquisition and retrieval of the task depending on its duration and on the species used. Seventy-two hours of REM SD preceding training have not been reported to affect the learning of the task in rats (Silva et al., 2004a; Alvarenga et al., 2008), whereas 96 h REM SD does impair learning in mice (Silva et al., 2004a; Alvarenga et al., 2008). On the other hand, in both studies a detrimental effect of REM SD have been documented on the consolidation and retrieval of the PM-DAT during the test session, both when it preceded and followed the training (Silva et al., 2004a; Alvarenga et al., 2008).

The experimental paradigms presented above are instances of delay conditioning: the US follows the CS at some specific delays or the CS continues to be present during the CS-US interval. Unlike delay conditioning, trace conditioning requires the CS to be a discrete event and to be separated by a temporal gap from the US (Pavlov, 1927). Hippocampus has a prominent role in learning

tasks that require temporal processing of information (McEchron et al., 1998; Runyan and Dash, 2005). Importantly, trace conditioning, especially in the form of eyeblink reflex conditioning, has been widely investigated as a model of associative learning and declarative memory in rodents (Christian and Thompson, 2003). To our knowledge, only one study explored the effects of SD on trace conditioning (Chowdhury et al., 2011). The authors reported that 6 h of total SD by gentle-handling soon after training impaired rats' performance in a task of trace-conditioned memory, measured as the number of head entries into a fruit juice dispenser (US). The authors therefore concluded that SD given after training was able to impair the encoding of trace memory.

OBJECT RECOGNITION

The "novel object recognition" (NOR) test exploits the rodents' spontaneous preference for novelty to measure recognition memory. In the typical paradigm, animals are presented and allowed to familiarize with a set of behaviorally meaningless objects for a brief amount of time (e.g., 5–10 min). After a variable delay (from a few minutes to one or more days) the object set is presented again, but with one of the familiar objects replaced with a novel one. The time spontaneously spent by the animal exploring the novel object, relative to the familiar one(s) is taken as a measure of recognition of the previously seen objects. It has been proposed that memory for objects in rodents is similar to episodic memory in humans (Dere et al., 2004) and relies upon peri- and post-rhinal regions rather than the hippocampus (Winters et al., 2004), if the spatial location of the objects is not changed.

Total SD by GH for 6 h following the acquisition phase in the NOR test severely impairs object and location recognition in a complex scene in a later test (Palchykova et al., 2006; Halassa et al., 2009). Interestingly, when SD was administered 6 h after the acquisition phase (thus allowing the animals to sleep undisturbed afterwards), no discrimination deficits were observed (Palchykova et al., 2006). Moreover, when GH occurred during the dark (activity) phase, no recognition memory deficits were observed in the subsequent test session (Palchykova et al., 2009).

Assessment of episodic memory in rodents relies on paradigms in which animals are required to bind the memory of an object to a spatio-temporal context (Kart-Teke et al., 2006). In such tasks, after few training trials, animals learn to remember not only the identity and location of the previously encountered object, but also the temporal order of object presentation. It has been reported that a brief (80 min) total SD by GH, following the acquisition phase in a modified NOR test, impairs rats' ability to consolidate and retrieve the memory for space, identity, and temporal order of presentation of an object (Inostroza et al., 2013).

THE EFFECTS OF SLEEP RECOVERY

A period of recovery sleep after SD is obviously considered beneficial to cognitive function. Relevant questions, however, remain open on the nature of such benefits.

In an instrumental learning study (Dubiela et al., 2005), one group of rats was deprived of REM sleep for 96 h, trained in an inhibitory avoidance apparatus immediately afterwards, and tested 30 min after the end of training. Performance was compared to that of rats allowed to recover for 24 h after REM SD and before training. The rate of acquisition was not affected by SD or by sleep rebound, but performance during the test differed across groups. In particular, SD animals entered the aversive chamber after a significantly shorter latency, compared to non-deprived rats, and animals allowed a period of sleep recovery showed an intermediate memory performance (Dubiela et al., 2005, 2010). The findings suggest that the avoidance deficits observed as a consequence of SD suffered immediately before training were due to inefficient/incomplete encoding of the memory association between the environment and the aversive experience.

Sleep recovery also improves recall of information acquired prior to the SD epoch. In a recent study (Wang et al., 2009) rats were subjected to 72 h REM-selective SD at the end of a 5-day training period in the MWM. The ability to recall the trained platform location was found to be impaired when tested immediately after SD, and the deficit was regarded as disruption of hippocampus-dependent spatial memory. When animals, however, were allowed to sleep for 18 h before the test, their performance turned out similar to that of control animals. This indicates that REM SD had no effect on the long-term memory trace consolidated over the training days. Rather, the results should be ascribed to deficits in *memory recall*, arguably due to the general disturbance of SD on cognitive function.

In both scenarios, SD appears to interfere with the animal's cognitive set rather than on the memory trace. In the former case, impaired cognition prevents encoding, while in the latter it prevents retrieval.

CAN DRUGS REVERSE SD-INDUCED MEMORY IMPAIRMENTS?

As described in the previous paragraphs, a transient memory deficit can be quite easily obtained in both rats and mice by means of SD. One of the crucial distinctions between the available paradigms is represented by the temporal relationship between the SD procedure and the animal's learning/testing experience (**Figure 1**). Depending on the adopted protocol, the observed memory deficits could be explained as the result of either impaired acquisition/consolidation or information retrieval. Consequently, drug-related effects would have to be attributed to mechanisms involving different aspects of the mnemonic experience.

The possibility to reverse SD-induced cognitive impairments through the administration of a variety of compounds (e.g., cognitive enhancers such as modafinil and donepezil, stimulants such as caffeine and nicotine, melatonin, vitamin E, etc.) has received some attention in recent years. In human healthy volunteers, positive effects of modafinil and caffeine in maintaining attentional and executive control performance during prolonged SD have been demonstrated (Wesensten et al., 2005; Killgore et al., 2009). On the other hand, chronic administration of donepezil (5 mg) has been shown to improve subjects' performance both in a visual short term memory and in a verbal episodic memory task after 24 h of total SD (Chuah et al., 2009; but see Dodds et al., 2011).

The effects of the non-amphetamine, wake-promoting agent modafinil in restoring memory performance after SD have been

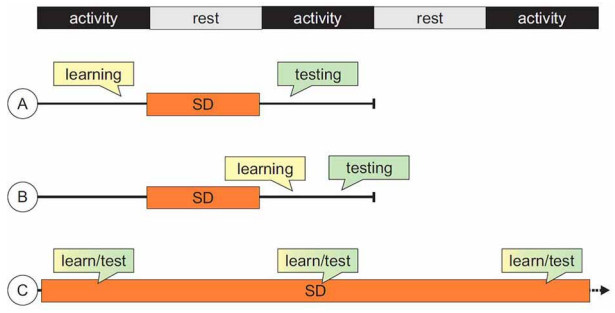

FIGURE 1 | Summary of typical sequences of events in studies of cognitive impairments induced by sleep deprivation (SD). (A) SD follows the acquisition or training phase and precede testing session(s); (B) both training and testing sessions are carried out *after* the SD epoch; (C) for tasks in which performance can be measured during training itself, sequences of training/SD can be administered repeatedly, allowing the assessment of long-term learning curves.

extensively studied both in mice and rats. The decrement in performance in the RAWM after 3 days of REM SD in rats was prevented by administration of modafinil for 1–3 days (He et al., 2011). Beneficial effects of modafinil administered prior to training were reported also in maintaining the inhibitory avoidance response after 96 h of REM SD in rats (Moreira et al., 2010). Modafinil, administered after training and SD, restored spatial working memory performance in a spontaneous alternation task (Pierard et al., 2007) and compensated contextual memory deficits in mice after 10 h of total SD (Pierard et al., 2011). Acute nicotine treatment also prevented retention deficits in a RAWM after 24 h of REM SD in rats (Aleisa et al., 2011). Significant pro-cognitive effects of melatonin (Chang et al., 2009) and vitamin E (Alzoubi et al., 2012) on memory impairments induced by SD in rats have also been reported.

Experiments testing the efficacy of drugs in reversing the transient memory deficits caused by SD could thus provide reliable models in drug-discovery preclinical studies for the treatment of dementia-related cognitive decline. To date, treatments of AD and other dementias rely on the administration of anticholinesterase therapy (e.g., donepezil, rivastigmine, galantamine) or NMDA receptor antagonists (i.e., memantine) whose effects at a symptomatic (cognitive and behavioral) level and as neuroprotective agents are still under debate (Bullock, 2002; Francis et al., 2005). The availability of rodent models of transient memory impairments would allow to specifically test the efficacy of drugs in ameliorating memory deficits and to study the duration of such improvements. In this context, the choice of the appropriate protocol of challenge, drug administration, and memory test deserves special attention not only in view of a translation to human patients, but also in relation to the specific aspect of memory impaired by SD (for example, learning impairment *vs.* deficit in the retrieval of previously acquired information).

CONCLUDING REMARKS

The available evidence here reviewed indicates that appropriately conducted SD protocols reliably cause memory deficits in rodents. Behavioral control conditions included in many studies offer convincing dissociations in support of the notion that SD-induced cognitive impairments are not simply the result of generic fatigue, stress, or lack of motivation. Rather, the results of properly controlled experiments suggest that deficits are caused by the disruption of specific high-level cognitive functions.

Moreover, certain functions are more prone to being disrupted by SD than others, and consistent experimental results may be difficult to obtain for some cognitive domains. For example, as previously reported, SD exerts a marked negative effect on hippocampus-dependent spatial memory consolidation tested in the MWM as well as on contextual fear memory tested in the shuttle-box, whereas no effect of sleep loss has been reported on cued fear memory. Interestingly, lack of significant deficits and even paradoxical performance improvements of SD have been also documented (Marti-Nicolovius et al., 1988; Smith, 1996; Tian et al., 2009).

Importantly, we find that the majority of studies employ SD as a tool to investigate the neurobiological mechanisms subserving sleep and sleep loss. Thus, the assessment of SD-induced cognitive deficits, rather than being the focus of the study, is often treated as an internal control of the success of the SD procedure, in view of subsequent molecular and neurophysiological evaluations. As a consequence, less attention is devoted to the subtleties of the experimental manipulations and consequent cognitive alterations. Thus, protocols that interfere with information encoding are used almost interchangeably with protocols that challenge information retrieval. The importance of dissociating the various cognitive processes underlying task performance is central when testing the efficacy of pro-cognitive drugs. For example, a different impairment is caused by protocols in which SD is administered *after training* but *before testing* and protocols in which SD *precedes* both learning and performance measurements, or in which epochs of SD are intermixed with training/test sessions (**Figure 1**). And yet, the 3 paradigms have been used to obtain "memory deficits" without any further connotation of the concept. Overall, the available data indicate that, while inducing memory deficits by SD in laboratory rodents is relatively straightforward, conceptualization of the effects of the different available protocols requires further testing and critical interpretation.

An example of a high-priority experimental use of SD is the preclinical testing of cognitive enhancers for mild cognitive impairment (MCI) and AD. MCI is considered to be a transitional phase between normal ageing and clinically probable, very early AD (Petersen, 2004). Currently available transgenic models of AD dementia have so far failed to fully replicate the phenotype of the human disease. For example, not all amyloid precursor protein transgenic mice become cognitively impaired, despite the presence of abundant plaques (Westerman et al., 2002). On the other hand, there are reports of cognitive impairment naturally occurring in aged laboratory rodents (Barnes and McNaughton, 1985; Gallagher et al., 1993), especially in the memory domain, but with striking interindividual variability in performance (Gallagher et al., 1993, 2003). It may be interesting, in principle, to apply SD protocols to transgenic animals and to measure the severity of subsequent memory impairments in order to quantify the additional costs, if any, of pathological changes

and behavior challenge. SD as cognitive challenge offers several benefits over other experimental procedures, in that its effects are transient, it is relatively easy to administer in a standardized fashion, it avoids pharmacological manipulations of cognition (drug-induced deficits). SD as cognitive challenge may therefore provide a promising preclinical model of MCI and a useful tool to study cognition enhancing drugs. Despite its inherent limitations and the specific concerns raised above, SD may prove especially successful in the context of translational research by allowing direct comparisons between preclinical studies and investigations in humans: whatever role sleep and sleep loss may ultimately play in cognition, such role is conserved between rodents and humans.

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Neuroimaging, cognition, light and circadian rhythms

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In humans, sleep and wakefulness and the associated cognitive processes are regulated through interactions between sleep homeostasis and the circadian system. Chronic disruption of sleep and circadian rhythmicity is common in our society and there is a need for a better understanding of the brain mechanisms regulating sleep, wakefulness and associated cognitive processes. This review summarizes recent investigations which provide first neural correlates of the combined influence of sleep homeostasis and circadian rhythmicity on cognitive brain activity. Markers of interindividual variations in sleep-wake regulation, such as chronotype and polymorphisms in sleep and clock genes, are associated with changes in cognitive brain responses in subcortical and cortical areas in response to manipulations of the sleep-wake cycle. This review also includes recent data showing that cognitive brain activity is regulated by light, which is a powerful modulator of cognition and alertness and also directly impacts sleep and circadian rhythmicity. The effect of light varied with age, psychiatric status, *PERIOD3* genotype and changes in sleep homeostasis and circadian phase. These data provide new insights into the contribution of demographic characteristics, the sleep-wake cycle, circadian rhythmicity and light to brain functioning.

Keywords: sleep, circadian, light, non-image-forming, non-visual, fMRI, cognition, inter-individual differences in sleep-wake regulation

INTRODUCTION

Cognitive brain responses and performance vary between and within individuals. In recent years, there has been a growing interest in the contribution of circadian rhythmicity and sleep-wake regulation to the within and between subject variation.

Typically, wakefulness and its associated cognitive processes are maintained for 16 continuous hours before sleep is initiated for about 8 h. This sleep-wake alternation is regulated by two mechanisms: the circadian and the homeostatic processes (Borbély, 1982; Daan et al., 1984). There is evidence that the impact of the interaction between these two processes is not linear such that variations in performance and brain function are particularly pronounced when wakefulness is extended into the biological night, when the combined influence of circadian and homeostatic processes is particularly negative for cognition (Dijk et al., 1992; Wyatt et al., 1999, 2004; Cohen et al., 2010). Importantly, time-of-day variations in cognitive performance differ between individuals, and particularly during the biological night, suggesting differences in the interplay between circadian and homeostatic processes. Moreover, light has traditionally been related to the circadian clock, but also conveys a direct (exogenous) stimulating signal that impacts on alertness and cognition (Lockley et al., 2006; Chellappa et al., 2011). Furthermore, inter-individual differences in the sensitivity to the impact of light are also starting to emerge (Vandewalle et al., 2011a; Chellappa et al., 2012).

The aim of the present review is to summarize recent neuroimaging studies providing the first neural correlates of the endogenous and exogenous regulation of sleep, wakefulness and cognition. We first describe the basics of sleep/wakefulness regulation. Next, we present functional neuroimaging studies describing variations of subcortical and cortical cognitive brain activity, during a normal waking day and following acute sleep deprivation. To investigate interindividual differences in sleep-wake regulation, chronotype and a polymorphism in *PERIOD3* (*PER3*) were used. We then focus on the impact of light on cognitive brain activity, and its interaction with circadian phase and sleep need. In the last section, a plausible scenario of the brain mechanisms through which sleep homeostasis, circadian rhythmicity and light affect cognition is presented.

COGNITIVE BRAIN FUNCTION IS TEMPORALLY ORGANIZED BY TWO INTERACTING PROCESSES

More than 30 years ago, the two-process model by Borbély and colleagues (Borbély, 1982; Daan et al., 1984) conceptualized sleep-wake regulation, by the interaction of a circadian and a homeostatic process. Sleep homeostasis is characterized by an increase or dissipation of sleep pressure, as wakefulness extends or sleep progresses, respectively, and is almost exclusively dependent on sleep-wake behavior. The mechanisms underlying this hourglass-like process are still debated, but animal research suggests that it arises from a use-dependent local augmentation

of sleep-promoting substances (adenosine (Basheer et al., 2004) and cytokines (Krueger, 2008)), from an increase in extracellular glutamate level (Dash et al., 2009), and/or from an experience-dependent increase of average brain synaptic strength, excitability and size during wakefulness (Vyazovskiy et al., 2008; Bushey et al., 2011). Other molecular markers of sleep loss have been identified in rodents (Franken and Dijk, 2009), while human polymorphisms have been associated with difference in sleep regulation [e.g., *PERIOD3 (PER3)* (Viola et al., 2007), *Adenosine Deaminase (ADA)*, *Adenosin A2a receptor (ADORA2A)*, *Brain Derived Neurotrophic Factor (BDNF)*, *Catechol-O-Methyltransferase (COMT)*, *human leukocyte antigen (HLA)*, (Goel and Dinges, 2011), *dopamine transporter (DA)*, (Valomon et al., 2014), *ABCC9* (Allebrandt et al., 2013); for review see (Landolt, 2011)]. At the macroscopic scale, the electroencephalogram (EEG) provides the best established markers of sleep need and intensity: slow wave activity (SWA; 0.5–4 Hz) during Non-Rapid Eye Movement (NREM) sleep (Dijk et al., 1987, 1997), and theta activity (4–8 Hz) during wakefulness (Cajochen et al., 2002). Such increases are particularly marked over frontal EEG derivations, the frontal cortex being particularly sensitive to the sleep pressure (Cajochen et al., 1999a). Besides global increases, SWA changes are also detected locally in areas most implicated in the task previously performed during wakefulness (Kattler et al., 1994), likely reflecting synaptic changes (Huber et al., 2004; Hung et al., 2013).

Behaviorally, increased sleep pressure is associated with a deterioration of cognitive performance, a decrease in alertness and an increase in sleepiness (Dijk et al., 1992; Wyatt et al., 1999; Lo et al., 2012). However, cognitive performance and its associated brain activity do not linearly decrease with increasing amount of time spent awake. This shows that a second, circadian regulation process impinges on cognition. The circadian signal is defined as a near-24 h endogenous, self-sustained oscillator, which determines the timing of the rest-activity cycle and of most physiological processes in synchrony with the environmental light-dark cycle. It is controlled by the suprachiasmatic nucleus (SCN), located in the anterior hypothalamus, also known as the circadian master clock (Moore, 2007).The circadian signal increasingly promotes wakefulness during the day, opposing the progressive accumulation of sleep pressure. It reaches a maximum level, in the so called wake-maintenance zone, in the evening (typically between 8 PM and 10 PM for an 11 PM–7 AM habitual sleep episode), preventing us from falling asleep despite the high need for sleep (Strogatz et al., 1987; Dijk and Czeisler, 1994, 1995). Once passing into the biological night, the circadian signal turns into a sleep-promoting signal, which increasingly opposes the dissipation of homeostatic sleep pressure during sleep, allowing a consolidated 8 h sleep episode. Although still putative, a sense of this circadian sleep-promoting signal can be found in the regulation of REM sleep and sleep spindles, which are most prominent at the end of the night (Dijk and Czeisler, 1995).

In humans, core body temperature (CBT) circadian profile is probably the closest to the dynamics of the circadian signal promoting wake/sleep. CBT progressively increases during the day to peak in the evening (at around 10 PM), before initiating a progressive decrease until the end of the night (at around 6 AM) (Dijk and Czeisler, 1995). Other gold-standard markers of the circadian process are melatonin and cortisol levels (Czeisler et al., 1999). The onset of melatonin secretion, a hormone signaling the circadian night, coincides with the end of the wake-maintenance zone and CBT maximum. Melatonin secretion increases until 2–3 h prior to CBT minimum. The well-known increase in cortisol upon awaking is considered as a marker of the end of the putative sleep-promoting zone and, being activating, has been suggested to provide a gate for the transition between sleep and wakefulness (Czeisler and Gooley, 2007).

The interplay between the circadian and homeostatic processes not only determines sleepiness and alertness levels, but also affects higher order cognitive functions (Dijk et al., 1992). During a normal waking day, the increase in homeostatic sleep pressure and deterioration in brain activity are counteracted by the circadian alerting signal. However, when wakefulness is extended into the biological night, the circadian system no longer opposes the high need for sleep, and cognitive performance is jeopardized, most strongly at the end of the night when the circadian signal maximally favors sleep (Dijk and Archer, 2010). Following chronic sleep restriction, which is common nowadays, the circadian signal cannot efficiently oppose abnormally high sleep pressure and maintain adequate performance already during the day. In addition, if wakefulness is extended into the biological night following chronic sleep restriction, the negative impact of acute sleep deprivation on cognitive performance is exacerbated (Lo et al., 2012).

NEURAL CORRELATES OF TIME-OF-DAY CHANGES IN BRAIN FUNCTION

Ten years ago, a positron emission tomography (PET) study investigated changes in brain glucose metabolism between morning and evening acquisitions (Buysse et al., 2004). Compared to the morning, evening quiet wakefulness was associated with increased metabolism in hypothalamic and brainstem structures, putatively encompassing several sleep/wake or arousal promoting nuclei. Decreased metabolism was also found at the cortical level in the temporal and occipital lobes (Buysse et al., 2004). Yet, a more recent PET study suggested no significant difference in metabolism (glucose and oxygen consumption) between morning and evening measurements (Shannon et al., 2013). However, using resting state fMRI data, changes in functional connectivity between the medial temporal lobe (MTL) and the rest of the brain were detected between morning and evening measurements (Shannon et al., 2013). In the morning, bilateral MTL regions were mainly functionally connected to local areas, while their connectivity spread cortically in the evening, in a set of regions important for memory consolidation. Since these effects did not appear to be affected by the length of prior wakefulness (they were unchanged following sleep deprivation), the authors speculated that these changes may reflect aspects of memory consolidation recurring on a daily basis. Similarly to the numerous studies investigating the impact of sleep deprivation on cognitive brain function (for review see Chee and Chuah, 2008), the latter two studies were in fact not designed to disentangle the changes associated with circadian and sleep homeostatic processes, or their interaction. In addition, the conditions, in which subjects

were maintained in between morning and evening PET and fMRI measures, were not carefully controlled for.

Critically for this review, there is evidence that there is a large inter-individual variability in the cognitive reaction to sleep loss. Some individuals are more resilient than others (Frey et al., 2004; Van Dongen et al., 2004; Viola et al., 2007), suggesting that additional factors affect the interplay of circadian and homeostatic processes. In the next two sections, we focus on three recent neuroimaging studies, which used known markers of interindividual differences in circadian and homeostatic sleep-wake regulation, and carefully controlled experimental conditions. These studies investigate interindividual variability in the temporal organization of cognitive brain function, during a normal sleep-wake cycle as well as after sleep deprivation.

CHRONOTYPE AND TIME-OF-DAY INFLUENCE ON BRAIN ACTIVITY SUSTAINING BASIC FORMS OF ATTENTION AND EXECUTIVE PROCESSES

Schmidt et al. (2009, 2012) used extreme chronotypes and their differences in sleep-wake regulation in order to investigate variations in brain activity. Differences in circadian timing preference are expressed in favorite periods of diurnal activity, such as working hours, and in specific sleep habits (Taillard et al., 2003), reflecting individual's particular chronotype. Extreme morning types are located at one end of the continuum. They show a marked preference for waking up very early, and find it difficult to remain awake beyond their usual bedtime. At the opposite end, extreme evening types prefer to go to bed in the late hours of the night, and often find it extremely difficult to get up in the morning. Extreme chronotypes are "phase-shifted" according to their circadian rhythmicity, that is, the peaks and troughs of their physiological circadian markers (CBT, melatonin) occur earlier or later in relation to the external clock time (Kerkhof and Van Dongen, 1996; Duffy et al., 1999; Baehr et al., 2000; Bailey and Heitkemper, 2001; Duffy et al., 2001; Mongrain et al., 2004). Furthermore, chronotypical differences have also been observed in the phase relationship between sleep-wake cycle and underlying circadian rhythms (phase angle of entrainment) (Duffy et al., 1999; Baehr et al., 2000; Liu et al., 2000). However, the finding of phase-angle differences in chronotypes has not been systematically replicated (Bailey and Heitkemper, 2001; Mongrain et al., 2004). Accumulating evidence also suggests that chronotypes differ in their homeostatic sleep regulation. Morning types have been reported to show a faster build-up (Taillard et al., 2003) and dissipation rate of homeostatic sleep pressure. Likewise, morning types also tend to begin their sleep episode with higher SWA levels in anterior brain areas (Mongrain et al., 2006b). Chronotypes have thus been shown to differ in circadian and homeostatic sleep-wake regulatory processes, and constitute an appropriate tool to investigate the interaction of circadian and homeostatic processes under normally entrained day-night conditions.

In both papers of Schmidt et al. (2009, 2012), extreme morning and evening types underwent a morning and an evening fMRI session, which was timed according to unconstrained preferred sleep and wake times, respectively 1.5 h and 10.5 h after wake-up time. During the fMRI sessions, two successive tasks were administered: the psychomotor vigilance task (PVT; Dinges and Powell, 1985) and the Stroop task (Stroop, 1935). The PVT records reaction times (RT) to random occurrences of a simple visual cue and probes sustained attention, i.e., the ability to maintain attention over prolonged periods of time (Dinges and Powell, 1985) which is considered as a fundamental form of attention onto which many other cognitive processes build (Raz and Buhle, 2006). The PVT has been repeatedly used to show circadian and sleep homeostatic influences on cognition (Lim and Dinges, 2008). Schmidt et al. (2009) focused on two PVT measures: global alertness, corresponding to trials with intermediate RT, and optimal alertness, associated with fastest RTs, phasically occurring when the participant is able to recruit attentional resources above and beyond the normal level set by global alertness. Only small differences between chronotypes were observed in the morning session, when homeostatic sleep pressure is low. By contrast, in the evening session, when sleep pressure is higher and the circadian signal strongly promotes wakefulness, global alertness was associated with increased thalamic responses in morning as compared to evening types. The observed response was located in a dorsomedial part of the thalamus compatible with the anterior pulvinar. Interestingly, the pulvinar has been showed to actively regulate cortical activity based on attention demand (Saalmann et al., 2012). Pulvinar activation has also been related to arousal level following total sleep deprivation (Portas et al., 1998), noradrenaline administration (Coull et al., 2004), as well as during and following light exposure as an external activating factor (Vandewalle et al., 2006). The result of Schmidt et al. (2009) suggests therefore that, with higher homeostatic sleep pressure and compared to evening types, morning types are under more demanding condition and recruit relatively more the pulvinar for maintaining a normal global alertness level. In addition, maintaining optimal alertness in the subjective evening was associated with larger responses, in evening compared to morning chronotypes, in a brainstem region compatible with the locus coeruleus (LC; Schmidt et al., 2010), and the suprachiasmatic area (SCA), i.e., an anterior hypothalamus region that encompassed the SCN (**Figure 1B**). The LC is the major source of norepinephrine and has widespread thalamic and cortical connections, so that it can potentially modulate higher-order cognitive functions (Aston-Jones, 2005). LC and SCN are two connected structures involved in the generation of the circadian arousal signal, which could regulate cognitive output during a waking day (Aston-Jones, 2005). Thus, the known improved cognitive ability of evening chronotypes towards the end of a normal waking day may result from their ability to recruit these interacting subcortical structures above normal levels.

When analysing polysomnographic data, SWA of the first NREM cycle was higher and dissipated faster in the course of the night in morning types, in agreement with previous findings (Mongrain et al., 2006b; **Figure 1A**). An independent regression analysis revealed that SCA activity related to optimal task performance was inversely proportional to SWA of the first NREM cycle (**Figure 1C**). This observation suggests a negative relation between homeostatic sleep pressure and response associated with optimal alertness during a PVT within the SCA, putatively encompassing the circadian master clock. Interestingly, data obtained in rodents similarly point to an impact of homeostatic

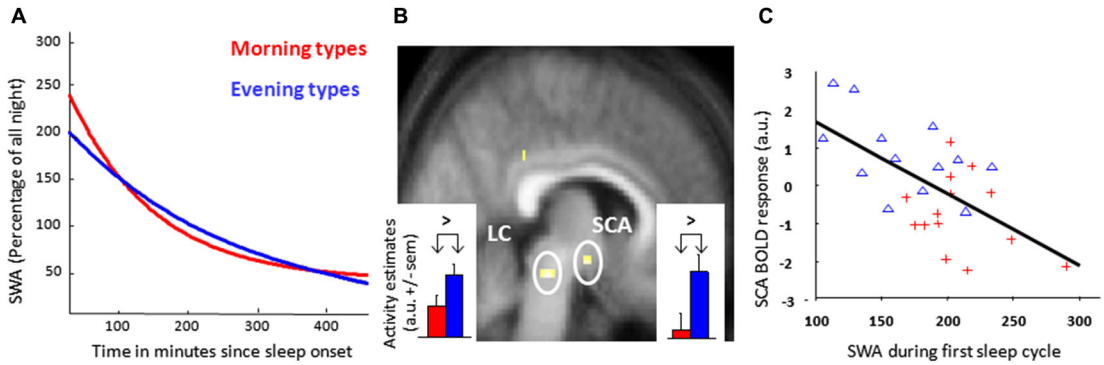

FIGURE 1 | Morning and evening chronotypes differ in their brain responses to an attentional task and SWA. (A) Exponential decay function adjusted on relative SWA in sleep cycles (NREM sleep) measured from the central frontal derivation for all-night EEG of the night preceding the evening scan acquisition. **(B)** Increased task-related response in the dorsal pontine tegmentum and the anterior hypothalamus, compatible with the locus coeruleus (LC) and suprachiasmatic area (SCA) respectively, in evening as compared to morning chronotypes during the subjective evening for optimal sustained attention during the performance of a Psychomotor Vigilance Task (PVT). Corresponding activity estimates (arbitrary units—a.u. +/− sem) are displayed for event indicators of fast reaction times. **(C)** Regression analysis of the relation between estimated blood oxygen level-dependent (BOLD) responses during optimal task performance in the SCA region and the amount of SWA during the first sleep cycle in the preceding night ($r = 0.54$, $p < 0.05$, $n = 27$). Red crosses: morning types, blue triangles: evening types. [Copied with permission from Schmidt et al. (2009); Cajochen et al. (2010)].

sleep pressure markers on electrical activity within the SCN (Deboer et al., 2003, 2007). Globally, the results suggest that evening types are more able to recruit arousal-promoting brain structures to maintain optimal alertness, even with increasing homeostatic sleep pressure. It may be assumed that performance of morning types would deteriorate in the evening, through a negative impact of sleep pressure on the master circadian clock that is not compensated by the increase in thalamic activation associated with global alertness. Conversely, it may be through a decreased ability of anterior hypothalamic activity to counteract sleep pressure that morning types undergo greater performance decrement.

The next logical question is whether sleep-wake regulatory processes similarly or differently affect more demanding executive processing. Schmidt et al. (2012) then turned to the second task of their protocol, the Stroop task, which probes interference by challenging continuous control over conflicting information. In this color-word task, subjects have to indicate as quickly as possible the color in which a word is printed while ignoring its meaning (e.g., incongruent item: word "blue" printed in red, congruent item: word "blue" printed in blue). By comparing congruent to incongruent items, the task allows the isolation of brain activity linked to cognitive interference. From the subjective morning to the subjective evening, evening types maintained or even increased interference-related responses in a set of brain areas playing a pivotal role in successful inhibitory functioning, whereas morning types presented decreased responses under the same conditions (Schmidt et al., 2012). Furthermore, in the evening, a regression showed that interference-related fMRI activity in the posterior part of the hypothalamus was negatively related to SWA of the first sleep cycle for morning types, whereas no significant correlation was found in evening types. This hypothalamic cluster was more posterior than in the PVT study, and was compatible with the lateral hypothalamus (LH), site of orexin (ORX) and melanin-concentrating hormone (MCH; Adamantidis and de Lecea, 2008), close to the location showing decreased gray matter concentration in narcoleptic patients (Draganski et al., 2002). These findings suggest that, in evening types, promotion and maintenance of appropriate cognitive interference abilities at the cortical level depend on posterior hypothalamus activity in the evening, when homeostatic sleep pressure is higher. In morning types, a relative weakness in the transmission of alerting signals from subcortical structures, e.g., the posterior hypothalamus, to the cortex could be the reason of decreased activity in interference-related brain structures from the morning to the evening hours.

PERIOD3 GENOTYPES, TIME-OF-DAY AND SLEEP-LOSS INFLUENCES ON COGNITIVE BRAIN RESPONSES

The following study we present used *PER3* genotype to investigate interindividual differences in the negative effect of sleep loss on brain activity. A primate-specific variable number tandem repeat (VNTR) polymorphism (4 or 5 repeats) in the coding region of the clock gene *PER3* is associated with individual preference of waking activity and sleep (Archer et al., 2003; Lázár et al., 2012), with $PER3^{5/5}$ showing morning preferences and $PER3^{4/4}$ evening preferences. Approximately 10% of the population is homozygous for the 5-repeat ($PER3^{5/5}$), whereas 50% present the 4-repeat ($PER3^{4/4}$). Although the two genotypes do not differ in circadian phase, $PER3^{5/5}$ seem to have a more rigid circadian control (Archer et al., 2008). Regarding sleep characteristics, $PER3^{5/5}$ have shorter sleep latency, more slow wave sleep (SWS) and more SWA, particularly in the first part of the night, both following a normal waking day or prolonged wakefulness, a profile similar to the one observed in morning types (Viola et al., 2007). During sleep deprivation, analysis of the waking EEG reveals a more rapid increase of theta/alpha activity and more frequent slow eye movements, a marker of inattention and drowsiness (Cajochen et al., 1999b) in $PER3^{5/5}$, compared to $PER3^{4/4}$ participants.

Furthermore, during the recovery night from sleep deprivation, the compensatory increase in SWS leads to a stronger suppression of REM sleep in *PER3*$^{5/5}$ (Viola et al., 2007), indicating a possible homeostatic regulation of SWS at the expense of REM sleep (Dijk and Archer, 2010). To summarize, both genotypes have an identical circadian oscillator but differ in the homeostatic regulation of sleep, with *PER3*$^{5/5}$ having an accelerated build-up of sleep pressure (Dijk and Archer, 2010). These data have been in part replicated in a nap protocol (Maire et al., 2014b) and in older people (Viola et al., 2012).

Regarding the impact of sleep deprivation on cognition, there also seem to be important divergences between genotypes. While performance remains similar between genotypes during a normal waking day, with a slightly larger decrement in *PER3*$^{5/5}$, during early-morning hours following acute sleep loss, *PER3*$^{5/5}$ have a worse decline in cognitive performance, particular so for executive tasks (Viola et al., 2007; Groeger et al., 2008). Furthermore, it has been shown that time-on-task decrement in performance observed during the realization of a PVT was also *PER3* genotype-dependent, with worse decrement for *PER3*$^{5/5}$ (Maire et al., 2014a). A recent simple model considered performance as a result of a non-linear interaction between the circadian and homeostatic signals, and matched differences between *PER3* genotypes (Dijk and Archer, 2010). The non-linearity of this interaction can be particularly sensed in the early morning hours, when the circadian sleep-promoting signal amplifies the differences in homeostatic sleep pressure, such that performance deteriorates disproportionally in *PER3*$^{5/5}$ individuals (Dijk and Archer, 2010; **Figure 2**). The latter model also explains why little behavioral differences were found between *PER3* genotypes that were allowed to sleep in the second half of the night during a sleep restriction protocol (Goel et al., 2009) (i.e., performance was not assessed during the second part of the night, when *PER3*-dependent effect are more pronounced).

Vandewalle et al. (2009a) studied normal volunteers prospectively recruited on their *PER3* genotype in an fMRI study. Each subject participated in two experimental segments, separated by at least 1 week, which were identical except for the presence or absence of sleep between the evening and morning fMRI sessions. The evening fMRI acquisition was scheduled 2 h before habitual bedtime, i.e., within the wake-maintenance zone, while the morning fMRI session was 1.5 h after wake time, after the putative sleep-maintenance zone. Thus, the morning and evening sessions differed with respect to both time awake and circadian phase, while morning sessions were scheduled at the same circadian phase and differed only for previous amount of time wake. In each session, participants performed an auditory working memory 3-back task (Cohen et al., 1997) in two consecutive recordings, once in darkness and once while exposed to light. Only the first recording will be considered here. The second will be summarized in the following section on the impact of light.

Analyses first focused on changes across a normal waking day and revealed that, in the evening relative to the morning session after sleep, *PER3*$^{5/5}$ participants showed reduced activation in the posterior dorsolateral prefrontal cortex (DLPFC) implicated in higher executive control (Koechlin et al., 2003), while *PER3*$^{4/4}$ did not show any significant changes. Comparing both morning sessions (low vs. high homeostatic sleep pressure) revealed an apparent double dissociation between the genotypes (**Figure 3**).

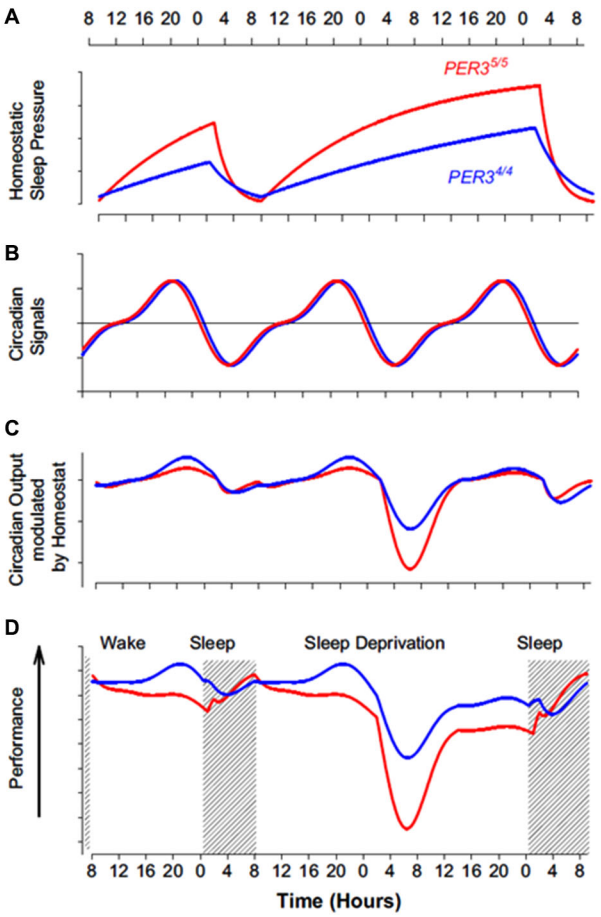

FIGURE 2 | Impact of the interaction between homeostatic sleep pressure and circadian process on cognitive performance in *PER3*$^{4/4}$ (blue line) and *PER3*$^{5/5}$ (red line) individuals. **(A)** As indicated by slow wave activity (SWA) measure, *PER3*$^{5/5}$ have a faster build-up during wakefulness and a quicker dissipation during sleep of homeostatic sleep pressure (based on data from Viola et al. (2007)). **(B)** Regarding circadian phase, *PER3*$^{4/4}$ and *PER3*$^{5/5}$ do not appear to differ, as indicated by melatonin, cortisol, and *PER3* mRNA measures (based on data from Viola et al. (2007)). Note that the circadian signal increasingly promotes wakefulness during the day (positive value, above horizontal line) and increasingly promotes sleep during the night (negative value, below horizontal line). **(C)** Theoretical modulation of the circadian signal by homeostatic sleep pressure in both *PER3* genotypes. The difference in homeostatic sleep pressure results in a limited difference in the output of this interaction during a normal waking day. The output of the interaction affects much more negatively wakefulness of *PER3*$^{5/5}$ in the absence of overnight sleep, particularly in the early morning hours when the circadian system maximally promotes sleep. **(D)** Composite measures of performance in both *PER3* genotypes, based on extended neurophychological test batteries (Viola et al., 2007). Performance profile closely follow theoretical interaction between circadian and sleep homeostasis processes depicted in **C**. This model could speculatively be applied to extremes morning and evening chronotypes, which also differ in term of homeostatic sleep pressure build up (Mongrain and Dumont, 2007) (but also they differ sometimes in term of circadian phase angle with sleep (Mongrain et al., 2006a)). [Copied with permission from Dijk and Archer (2010)].

PER3$^{5/5}$ volunteers showed decreased activations following sleep loss in a widespread set of areas involved in the ongoing task (Cohen et al., 1997; Collette et al., 2005, 2006), including temporal, parietal and occipital areas, and again bilaterally in the DLPFC. By contrast, *PER3*$^{4/4}$ participants recruited supplementary brain areas to perform the task in the ventro-lateral prefrontal cortex (VLPFC), temporal cortex, as well as a thalamic region compatible with the pulvinar. Comparisons between the morning session after sleep deprivation and the evening wake-maintenance zone session were then computed (large differences in the sleep/wake promoting circadian signal vs. intermediate differences in sleep pressure). Analyses revealed again increased compensatory activations in *PER3*$^{4/4}$ and decreased activations in *PER3*$^{5/5}$in the same cortical and subcortical regions than those observed when comparing morning sessions. Importantly, these activation profiles were even more pronounced than when comparing morning after sleep and morning after sleep deprivation sessions, especially in *PER3*$^{5/5}$ individuals. If sleep homeostasis was primarily responsible for differences observed, then differences should be reduced when comparing the morning session after sleep deprivation to the evening session after a normal waking day. Likewise, if the circadian signal was solely responsible for changes in cognitive brain responses, then a difference should only be present when comparing morning to evening sessions.

Taken together these data and those of Schmidt et al. (2009, 2010) confirm that the daily temporal organization of executive brain activity depends on the endogenous mechanisms regulating sleep and wakefulness, i.e., the interplay between sleep homeostasis and the circadian clock. A question, then asked by Vandewalle et al. (2011a) was how this daily temporal organization was affected in the presence of an exogenous factor impinging on sleep and wakefulness: light exposure. After a brief reminder of previous findings on the effect of light on brain function, we will summarize results obtained by investigating groups differing with respect to *PER3* genotype, age or psychiatric status.

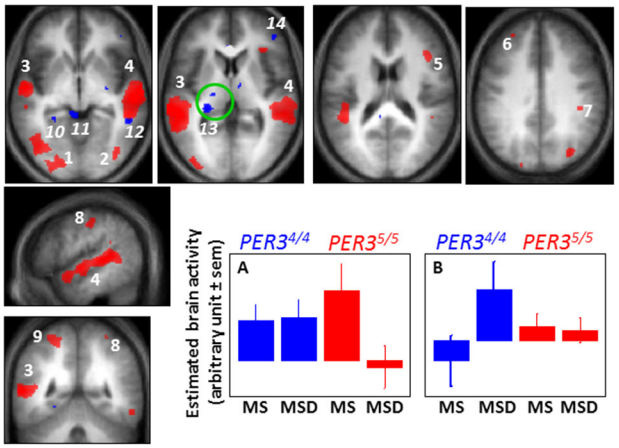

FIGURE 3 | Difference between *PER3*$^{4/4}$ and *PER3*$^{5/5}$ individuals in the sleep loss-induced changed in brain responses to a working memory task. When comparing brain responses to an auditory 3-back task in the morning after a night of sleep (MS 1.5 h of wakefulness) and in the morning after a night of sleep deprivation (MSD 25 h of wakefulness), *PER3*$^{5/5}$ individuals undergo marked decreases in activation in several brain areas of the occipital (1, 2) and temporal (3, 4) cortices, and of the dorsolateral prefrontal (5, 6) and parietal cortex (7–9), while *PER3*$^{4/4}$ individuals maintain brain responses in these areas (and do not present significant decreased activations in any brain regions). A representative profile of this brain activity change is displayed in panel **A** (similar profiles were observed for red areas 1–9). In contrast, when comparing the same sessions, *PER3*$^{4/4}$ individuals present increased activation (blue) in the parahippocampus (10), superior colliculus (11), temporal cortex (12), pulvinar (13), and ventrolateral prefrontal cortex (14), while no increased activation is observed in these regions in *PER3*$^{5/5}$ (and in any other brain regions). A representative profile of this brain activity change is displayed in panel **B** (similar profiles were observed for blue areas 10–14). A significant negative association was found between overnight change in brain response in the pulvinar (green circle) and self-reported daytime propensity to fall asleep in everyday life across all the subjects of the study (irrespective of genotype), further suggesting a central role for the pulvinar in wakefulness regulation. [Adapted with permission from Vandewalle et al. (2009a)].

LIGHT STIMULATES COGNITIVE BRAIN FUNCTION AND DIRECTLY AFFECTS SLEEP AND WAKEFULNESS

Light is necessary for image formation by the visual system, but is also essential for the regulation of numerous circadian, neuroendocrine, and neurobehavioral non-image-forming (NIF) functions, including the direct improvement of alertness and performance (Vandewalle et al., 2009b; Hatori and Panda, 2010; Schmidt et al., 2011; Bailes and Lucas, 2013). These NIF effects of light are mediated in part by a retinal photoreception system, which is distinct from the classical visual system. In addition to rods and cones, the NIF photoreception system recruits a novel class of photoreceptors, which consists in intrinsically photosensitive retinal ganglion cells (ipRGC) expressing the photopigment melanopsin and maximally sensitivity to blue light (ca 480 nm) (Berson et al., 2002; Dacey et al., 2005; Hatori and Panda, 2010; Schmidt et al., 2011; Bailes and Lucas, 2013). The ipRGCs play accessory visual functions (Ecker et al., 2010; Brown et al., 2012), but are deeply implicated in the NIF functions of light, which have therefore a sensitivity shifted toward shorter wavelength blue light. IpRGC constitutes the only channel though which light affects NIF functions (Guler et al., 2008); however, inputs from rods and cones are necessary to observe a complete response (Ruby et al., 2002). Melanopsin-expressing ipRGCs project to various brain structures, including hypothalamic, thalamic, striatal, brainstem and limbic structures (Hattar et al., 2006; Ecker et al., 2010). Importantly, ipRGCs have direct projections to the SCN. These widespread and numerous projections are an essential feature of the brain mechanisms, through which light can exert a potent and diverse impact on NIF functions.

Light can affect sleep, wakefulness and cognition indirectly, via its synchronizing/phase-shifting effects on the circadian clock. Critically, light also conveys a direct stimulating signal that affects sleep homeostasis (Cajochen et al., 1992; Altimus et al., 2008; Tsai et al., 2009; Chellappa et al., 2013), increases alertness and cognitive performance (Cajochen et al., 2005; Rahman et al., 2014), including during sleep inertia (Santhi et al., 2013). A series of neuroimaging studies investigated the brain mechanisms involved in the impact of light on cognition, using simple attentional task (oddball paradigm) (Vandewalle et al., 2006), more complex working memory task (n-back) (Vandewalle et al., 2007a, 2011b), and emotional tasks (Vandewalle et al.,

2010). In accordance with animal research, results of these studies are compatible with a scenario in which light would first influence subcortical structures involved in arousal regulation, before significantly affecting the cortical areas involved in the ongoing cognitive process (Vandewalle et al., 2009b; Vandewalle and Dijk, 2013; **Figure 4**). These subcortical structures include hypothalamus nuclei, possibly the SCN, the ventrolateral preoptic area, the dorsomedian hypothalamus, and/or the paraventricular nucleus of the hypothalamus (PVNH). The brainstem also appears to play a central role, within the LC or in other nuclei of the ascending activating system, while similarly, the pulvinar is repeatedly affected. Light impact on the latter two structures could greatly affect information flow within the cortex. If sufficient, light subcortical impact would then significantly affect the cortical area recruited for the ongoing cognitive process. Behavioral measures would only be significantly affected after prolonged light exposure, either because the light impact on cortical structures requires time to be transferred into behavior, or because behavioral measures are less sensitive than neuroimaging techniques, or probably both. Although photoreceptors are not as accessible in human as in animal models (e.g., genetic modifications are not possible), two recent studies provided compelling evidence that melanopsin-expressing ipRGCs were mediating the impact of light on cognitive brain responses (Vandewalle et al., 2013; Chellappa et al., 2014).

EFFECTS OF LIGHT ON COGNITIVE BRAIN RESPONSES DEPEND ON ENDOGENOUS PROCESSES AFFECTING SLEEP AND WAKEFULNESS

Aging is associated with important changes in the regulation of sleep and wakefulness. Briefly, sleep becomes more fragmented and the amount of SWS decreases, suggesting a less restorative sleep but a shallower build-up of sleep need (Klerman and Dijk, 2008; Carrier et al., 2009). The amplitude of the circadian signal also appears to be reduced in aging, as indicated by the reduced detrimental effect of night-sleep loss at the behavioral level, but also by earlier awakening during sleep (Daneault et al., 2013). Daneault et al. (2014) recently reported that, even if still present, the impact of light on brain responses was reduced in healthy older individuals (>60 y.o.), compared to younger individuals (<30 y.o.), when investigated after habitual sleep time (and therefore after the wake maintenance zone). Reduced impacts of light were observed notably within the insula, prefrontal cortex, amygdala, tegmentum and thalamus, which are key structures in the regulation of alertness and cognition.

In another study, patients suffering from Seasonal Affective Disorder (SAD—winter depression) were shown to present abnormal responses to emotional stimulation within the posterior hypothalamus, in a region compatible with the ORX/MCH LH or with the PVNH (Vandewalle et al., 2011b). Similar to aging, SAD is characterized by changes in the regulation of sleep and wakefulness (Cajochen et al., 2000), but with patients sleeping more and showing decreased motivation and mood.

The latter studies on SAD and aging could indicate that endogenous changes in sleep-wake regulation modify the impact of light on cognitive brain activity, or alternatively that changes in the impact of light contribute to changes in sleep-wake regulation. However, these studies did not include measures repeated over the 24 h day, so that no inference with respect to sleep homeostasis or circadian processes can be made. For instance, older individuals recruited more brain areas to perform the task independent of the light condition (Hedden and Gabrieli, 2004). The diminished impact of light is maybe due to the fact that older individuals were compensating

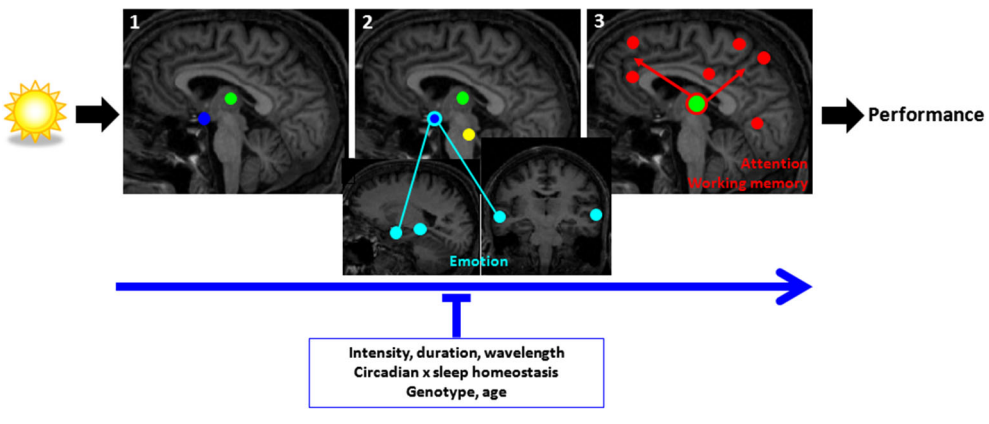

FIGURE 4 | Schematic representation of the brain mechanisms involved in the non-image-forming impact of light on cognitive brain responses. **(1)** Responses at light onset are found within the hypothalamus (blue) and pulvinar (green) (and amygdala and hippocampus, not shown); **(2)** within the first seconds of the exposure, responses are found mainly in subcortical and cortical structures involved in alertness regulation (hypothalamus, brainstem (yellow), pulvinar); **(3)** late responses are detected at the cortical level in areas involved in the ongoing cognitive process and can subsequently affect performance. For attention/working memory/executive task (red) a network of areas around the pulvinar and including prefrontal and parietal areas appear to mediate the impact of light on alertness and cognition. For emotional responses to vocal stimuli (light blue), the network involves the hypothalamus, amygdala, and voice-sensitive area of the temporal cortex. Light seems to have a swifter impact on emotional cortical responses than attentional/working memory/executive responses. The impact of light is stronger with higher intensity, longer duration, and shorter wavelength (blue) light exposures. Time of day and the associated changes in the interaction between circadian and sleep homeostasis signals and *PERIOD3* genotype modulate the impact of light. [Adapted with permission from Vandewalle et al. (2009b)].

for task difficulty and could not be helped as efficiently by light.

As already mentioned, Vandewalle et al. (2011a) also collected data including the *PER3* genotype and light exposure. The same participants were exposed to alternating blue and green light while performing a 3-back task. Light wavelengths were chosen to maximally stimulate the NIF photoreception system (blue) or the classical photopic system (green). Brain responses to the task under blue and green light were compared in the morning following sleep, in the evening wake-maintenance zone, and in the morning following sleep deprivation.

Results indicated that, in the morning shortly after sleep, blue light significantly enhanced brain responses to the task in prefrontal and parietal areas, as compared to green light (Vandewalle et al., 2011a). These blue light effects were only found in $PER3^{4/4}$ individuals. Surprisingly, no differential impacts of light wavelength were found in the evening wake-maintenance zone, indicating a relative decrease of the impact of light in that part of the circadian cycle. Finally, in the morning session after sleep deprivation, blue light significantly increased task-related brain activity. This blue light effect was observed again in the prefrontal and parietal cortices, but also in other areas, including the insula and the pulvinar (Vandewalle et al., 2011a). Importantly, in the morning after sleep deprivation, these effects of blue light were only observed in $PER3^{5/5}$.

A PUTATIVE SCENARIO OF THE BRAIN MECHANISMS INVOLVED IN THE INTERPLAY BETWEEN COGNITION, SLEEP-WAKE REGULATION AND LIGHT

Collectively, these neuroimaging studies investigating interindividual differences allow for a scenario speculating about the effects of homeostatic sleep pressure, the circadian signal and of light on cognition-related brain activity. It appears that the anterior hypothalamus, in a region compatible with the SCA, but also the ORX/MCH posterior LH, may constitute sites through which circadian and homeostatic processes interact (Schmidt et al., 2009, 2012) for the regulation of cognitive brain activity. Based on the data, one could also consider the hypothalamus as one of the first structures affected by light, within the SCA (Perrin et al., 2004), but also possibly in other nuclei such as the PVNH, dorsomedial hypothalamus (DMH) or LH in the context of an emotional task (Vandewalle et al., 2010, 2011b). Importantly, the hypothalamus, and particularly the SCN, is indirectly connected with the LC, a region of the brainstem that is the main source of norepinephrine (Aston-Jones, 2005), and is likely to be the brainstem region influenced by light in a nonvisual context (Vandewalle et al., 2007b). Both structures are highly implicated in the circadian regulation of sleep and wakefulness and have multiple connections to other relevant brain areas, including the thalamus, and cortex for the LC. Thus, the hypothalamus and LC could be the subcortical core that regulates the circadian alerting signal and the stimulating impact of light.

For more demanding cognitive challenges (e.g., late evening hours, sleep loss, higher order executive tasks), cortical regions seem to enter into play. When testing brain responses in the evening, both morning chronotypes and $PER3^{5/5}$ individuals were unable to maintain stable brain responses to cognitive inhibition or working memory tasks (Vandewalle et al., 2009a; Schmidt et al., 2012). In addition, when testing working memory in the morning following sleep deprivation, the recruitment of the lateral prefrontal cortex (LPFC) appears as a key factor for the maintenance of brain responses. $PER3^{5/5}$ are unable to maintain activation in DLPFC (Vandewalle et al., 2009a). By contrast, the recruitment of the VLPFC by $PER3^{4/4}$ under sleep deprivation may reflect a compensatory switch to a more appropriate cognitive strategy (Vandewalle et al., 2009a). The frontal lobe plays a major role in executive function and, according to a model, the VLPFC is important for cognitive control and is involved in complex neurobehavioral processes (Koechlin et al., 2003).

The pulvinar, which was more activated following sleep deprivation in $PER3^{4/4}$ individuals, appears also to play a central role in the ability to face sleep loss and circadian challenges, and may constitute a further subcortical site through which circadian and sleep homeostasis interaction affects cognition and alertness (Aston-Jones, 2005). This assumption is strengthened by supplemental analyses indicating a significant negative association between overnight change in task-related pulvinar brain responses and daytime propensity to fall asleep in everyday life across all the subjects of the study (i.e., irrespective of genotype) (Vandewalle et al., 2009a).

But how does the difference in the impact of light fit in this picture? Differences in the endogenous drive for wakefulness, or in compensatory mechanisms already in place, stand as a likely explanation. The combination of sleep loss and adverse circadian phase induces major reductions of activations across all parts of the cortex in $PER3^{5/5}$ individuals. Blue light appears to be effective in "rescuing" brain responses under these adverse circumstances. On the other hand, $PER3^{4/4}$ individuals are able to trigger endogenous compensatory brain mechanisms that maintain brain responses in the morning after a night without sleep, and blue light is less beneficial to them. The nonvisual impact of light would therefore provide more benefits to the genotype that is not able to maintain brain responses endogenously and is most challenged by the circadian and sleep homeostatic conditions. This would be the reason why a relative decrease in the stimulating impact of light was detected in both genotypes in the wake-maintenance zone, when the endogenous drive for wakefulness is maximum. In addition, $PER3^{5/5}$ individuals are more likely to be morning chronotypes and prefer to be active in the morning hours (Archer et al., 2003), so that in the morning following sleep they would be in optimal endogenous conditions to perform, and could not benefit as much from an external light stimulation. $PER3^{4/4}$ individuals, which represent 45–50% of the general population and are more likely to be evening chronotypes, would benefit more from light in the morning after a night of sleep. This hypothesis is in agreement with previous studies, which were carried out in the morning (after a normal night of sleep), and found a significant impact of light on brain responses in non-genotyped samples (Vandewalle et al., 2006, 2007a,b). Again the pulvinar, which was affected by blue light following sleep deprivation in $PER3^{5/5}$, also constitutes a possible interface between light impact and cortical cognitive brain responses.

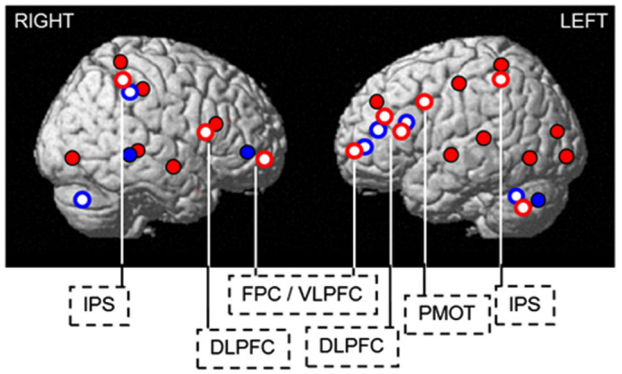

FIGURE 5 | Endogenous and exogenous regulation of sleep and wakefulness affects cognitive brain responses through overlapping pathways. Blue light increases cognitive brain responses in regions showing decreased activation (*PER3*$^{5/5}$) or compensatory recruitment (*PER3*$^{4/4}$) in darkness, following sleep loss. Blue solid: compensatory increase in activation in the morning hours after 25 h of wakefulness in *PER3*$^{4/4}$, found in the ventrolateral prefrontal cortex, temporal cortex, cerebellum, and thalamus (not shown). Red solid: decreases in activation in the morning hours after 25 h of wakefulness in *PER3*$^{5/5}$, observed in the occipital, temporal, parietal, and lateral prefrontal cortices. Red open: blue light-induced increase in activations after 25 h of wakefulness in *PER3*$^{5/5}$(thalamus not shown). Blue open: blue light-induced increase in activity after 1.5 h of wakefulness in *PER3*$^{4/4}$. DLPFC = dorsolateral prefrontal cortex; FPC / VLPFC = frontopolar / ventrolateral prefrontal cortex; IPS = intraparietal sulcus; PMOT = premotor cortex. [Copied with permission from Vandewalle et al. (2011a)].

CONCLUSION AND PERSPECTIVE

It is maybe remarkable that endogenous and exogenous mechanisms, regulating sleep and wakefulness, affect cognitive brain responses through at least partially overlapping pathways. These overlaying pathways become more obvious when overlaying differences in brain responses observed between *PER3* genotypes with and without light exposure (**Figure 5**). Thus we can gain insight into cognition regulation by manipulating wakefulness either endogenously or exogenously.

Overall, an "inverted U shape" profile, which would differ between individuals regarding chronotype or vulnerability to sleep loss, could fit with the results summarized in this review. In all individuals, accumulation of homeostatic sleep pressure would initially be associated with activation of arousal-related thalamic regions, and cortical areas, including prefrontal areas when higher cognitive processes are involved. The thalamus was indeed activated in morning types in the evening for a simple cognitive challenge. When the challenge becomes too adverse, brain responses are not maintained in these regions. This decreased-activation process begins at a different moment, depending on circadian and homeostatic characteristics of an individual. Morning chronotypes and *PER3*$^{5/5}$ seem to have a more rigid circadian control and perform better in the morning. Because of a faster homeostatic sleep pressure build-up, their cognitive resources would undergo a faster decrement, and they would benefit earlier of endogenous compensation, but suffer also from an earlier failure of these compensatory mechanisms. Instead, in the morning following a night without sleep, *PER3*$^{4/4}$, and putatively evening chronotypes, would engage compensation later and would therefore be able to maintain cognitive brain responses better under more adverse conditions, i.e., morning hours following sleep loss. Light would help more efficiently those individuals that are far from the apex of the inverted U-shape. This would also be valid for older individuals, which are already compensating for an important cognitive challenge, and would benefit as much from light.

The scheme remains highly speculative and has to be further explored by subjecting extreme chronotypes or older individuals to stringent sleep deprivation protocols, with or without light, or by increasing the sampling rate of neuroimaging assessment, under constant routine or forced desynchronize protocol, and particularly around critical time point such as the wake and sleep maintenance zone.

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Naps in school can enhance the duration of declarative memories learned by adolescents

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Sleep helps the consolidation of declarative memories in the laboratory, but the pro-mnemonic effect of daytime naps in schools is yet to be fully characterized. While a few studies indicate that sleep can indeed benefit school learning, it remains unclear how best to use it. Here we set out to evaluate the influence of daytime naps on the duration of declarative memories learned in school by students of 10–15 years old. A total of 584 students from 6th grade were investigated. Students within a regular classroom were exposed to a 15-min lecture on new declarative contents, absent from the standard curriculum for this age group. The students were then randomly sorted into nap and non-nap groups. Students in the nap group were conducted to a quiet room with mats, received sleep masks and were invited to sleep. At the same time, students in the non-nap group attended regular school classes given by their usual teacher (Experiment I), or English classes given by another experimenter (Experiment II). These 2 versions of the study differed in a number of ways. In Experiment I ($n = 371$), students were pre-tested on lecture-related contents before the lecture, were invited to nap for up to 2 h, and after 1, 2, or 5 days received surprise tests with similar content but different wording and question order. In Experiment II ($n = 213$), students were invited to nap for up to 50 min (duration of a regular class); surprise tests were applied immediately after the lecture, and repeated after 5, 30, or 110 days. Experiment I showed a significant ∼10% gain in test scores for both nap and non-nap groups 1 day after learning, in comparison with pre-test scores. This gain was sustained in the nap group after 2 and 5 days, but in the non-nap group it decayed completely after 5 days. In Experiment II, the nap group showed significantly higher scores than the non-nap group at all times tested, thus precluding specific conclusions. The results suggest that sleep can be used to enhance the duration of memory contents learned in school.

Keywords: learning and memory, sleep, memory consolidation, middle school

INTRODUCTION

There is an increasing interest in understanding the importance of sleep for academic performance in children and adolescents. The transition to adolescence comprises marked changes in sleep-wake patterns and underlying physiological factors, including typical behaviors such as delayed sleep phase syndrome (Gianotti et al., 1992; Louzada and Menna-Barreto, 2003, 2004; Crowley et al., 2007; Sousa et al., 2007; Beijamini and Louzada, 2012). In particular, eveningness is associated with later bedtime and wake-up time (especially on weekends), shorter time in bed during the week, longer weekend time in bed, irregular sleep-wake schedule, and subjective poor sleep (Crowley et al., 2007). Delayed phase syndrome is associated with extrinsic and intrinsic factors, such as electricity at home, technology and social context, as well as a strong relationship with pubertal maturation during development (Carskadon et al., 1993; Louzada and Menna-Barreto, 2003, 2004; Sousa et al., 2009). Together, delayed sleep phase syndrome, sleep habits and early starting times for school are responsible for promoting sleep deprivation in adolescents; and consequently, increase daytime sleepiness, attention and emotional problems, difficulties in memorization and concentration, and poor school achievement (Gianotti et al., 1992; Sousa et al., 2007; Beijamini and Louzada, 2012). This situation is not very different when it comes to children, whose typical school times and extracurricular activities reduce opportunities for daytime naps that are common among preschoolers. Furthermore, inadequate sleep in children has been related to attention deficits, irritability, emotional fragility and frustration (Dahl, 1996; Belísio et al., 2010). Changes in the sleep-wake cycle are thought to jeopardize school learning in two ways: they reduce the capacity for new learning, and impair the consolidation of what has already been learned (Louzada et al., 2008).

Some studies suggest negative health effects of napping among adults, such as a link to higher risk of diabetes, with different nap durations having different effects on health (Fang et al., 2013; Lucassen, 2013). On the other hand, multiple laboratory

studies indicate that sleep enhances neural plasticity and increments learning and memory (Maquet, 2001; Stickgold, 2005; Born et al., 2006; Walker and Stickgold, 2006; Ellenbogen et al., 2009; Diekelmann and Born, 2010). Declarative memories are specifically enhanced by slow wave sleep in both adults (Yaroush et al., 1971; Fowler et al., 1973; Plihal and Born, 1997) and children (Wilhelm et al., 2008; Prehn-Kristensen et al., 2009). Since slow wave sleep comprises most part of daytime naps (Bes et al., 1996), and declarative memories constitute the bulk of what is learned in school, naps have great potential to benefit school learning. In adults, daytime naps of 60–90 min enhance perceptual learning nearly to the same extent as an 8 h period of night time sleep (Mednick et al., 2003), and naps as short as 6 min have been reported to benefit declarative memory retention (Lahl et al., 2008). In infants, naps have been shown to enhance language learning (Gomez et al., 2006; Hupbach et al., 2009).

Despite the heightened interest on the cognitive role of naps, the actual effectiveness of naps in school learning remains largely unexplored. A recent study showed that school naps enhance learning in preschoolers (Kurdziel et al., 2013), but no information about adolescents is available. In the present study we investigated whether post-learning daytime naps can benefit the consolidation of declarative memories learned in school by adolescents, with a focus on a potential effect on memory duration.

METHODS

SUBJECTS

A total of 584 volunteer students (10–15 years old, mean age 11.3) from the 6th grade participated in the study, which involved 7 schools in the Brazilian city of Natal. The volunteers were healthy, did not present sleep disturbances, and did not use medication that affects the sleep/wake cycle. Students that failed any of those requirements were excluded from the analysis. The parents or legal guardians of the students signed consent forms with a description of the study procedures. The study was approved by the Research and Ethics Committee of the Onofre Lopes Hospital at the Federal University of Rio Grande do Norte (permit 336/09).

GENERAL EXPERIMENTAL DESIGN

Data collection took place entirely at school, without adjustment for the amount of sleep obtained by each student at home. In the morning (between 08:00 and 09:00) or in the afternoon (between 14:00 and 15:00) the students were exposed to a 15-min lecture on "vision and memory," a content that is typically unknown by the age group studied, and is absent from the standard curriculum of the 6th grade. The contents of the lecture were intentionally designed not to be relevant to the curriculum, thus avoiding interference with memories acquired before or during the experiment. The lecture was always presented by the same experimenter (NL) as a fixed sequence of images plus text projected on a screen. From a pedagogical point of view, the experimental lectures were designed to be highly engaging to the students in two ways: (i) the researcher (NL) always activated the students' schemata and elicited their prior knowledge, so that they could link it to the new input in an active fashion; (ii) the lecture was technology-mediated (a video projector was used) and attractive (high-resolution images of high perceptual salience), a rare practice in Brazilian public schools which may explain why the students typically reacted with enthusiasm and sometimes euphoria to the presence of the experimenter (NL). Immediately after the lecture, the students were randomly sorted into nap and non-nap groups. Students in the nap group were conducted to a quiet room with mats, received sleep masks and were invited to sleep. Students in the non-nap group proceeded to different interfering activities (see below). Finally, all the students were allowed to return to their regular classroom activities (**Figure 1**). The experimenter (NL) questioned subjects after their nap interval on whether they had slept, and/or dreamed. However, due to the degree of subjectivity and lack of reliability of this measure, these data were not included in the analyses and we decided to place all participants with access to sleep in the nap condition. Tests were administered without prior notice to the students at the same time of the lecture. Each student took two tests applied on different days. We did not seek to determine whether students studied the material at home, for we considered self-reporting too subjective. However, we took three measures to discourage the students to look further into the topics: students were not allowed to take notes during the lecture, the topics were off the curriculum, and the students were not aware that the researchers would come back days later to apply a test.

EXPERIMENT I

Students in the nap group were invited to nap for up to 2 h, and students in the non-nap group received a regular school class given by their regular teacher. A multiple-choice pre-test (PT; Supplementary Material 1) for lecture-related knowledge was applied immediately before the lecture, and a test comprising similar content but different in wording and question order was applied after 1, 2, or 5 days (T1, T2 and T5, respectively; Supplementary Material 2; **Figure 1A**). Therefore, each student was tested twice.

EXPERIMENT II

Students in the nap group were invited to nap for up to 50 min, which is the duration of a regular class period. The non-nap condition consisted of two sub-groups of equal size: one attended a regular English class given by their usual teacher; and another participated in an English class with games given by another experimenter (JW). A multiple-choice test was applied immediately after the lecture (T0), and identical follow-up tests were applied after 5, 30 or 110 days (T5, T30, or T110, respectively; **Figure 1B**; Supplementary Material 2). Therefore, each student was tested twice. Participants whose T0 scores were lower than 50% were discarded from the analysis.

STATISTICAL ANALYSES

Data from morning and afternoon tests were pooled. Significant differences were assessed using ANOVA followed by two-tailed Student's *t*-tests. One-way ANOVAs were used to test, within each contrast assessed (e.g., Experiment 2, T0 vs. T5) whether scores were higher when lectures were followed by naps, in comparison with waking activities. Two-Way ANOVAs were used to search for interactions between sleep (nap vs. no-nap) and time of the day (morning vs. afternoon) with regard to test scores, as well as

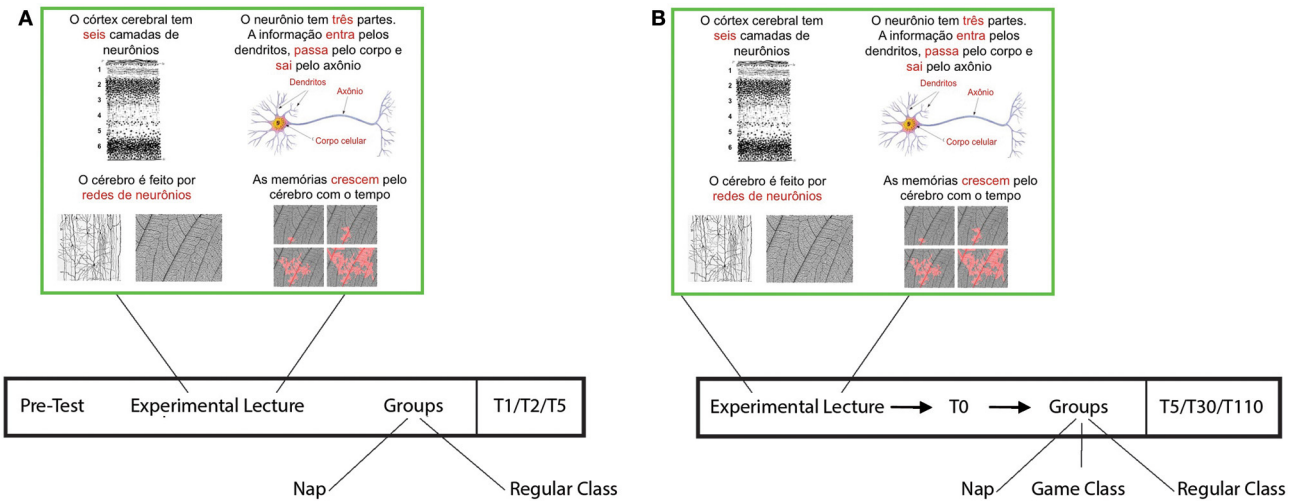

FIGURE 1 | Experimental design. Students were exposed to a lecture on novel declarative contents, sorted to nap or not to nap, and subjected to surprise tests after 1, 2, and 5 days. **(A)** Experiment I. **(B)** Experiment II. *Indicates significant differences with respect to the pre-test.

interactions between sleep (nap vs. no-nap) and time of testing (T0, T5, and T30) with regard to memory deterioration.

RESULTS

A total of 371 students (mean age 11.5 years old) participated in Experiment I. **Figure 2** illustrates score gains for both the nap and non-nap groups, calculated as the difference between post-class (T1, T2 ou T5) and pre-class (PT) scores. Both groups showed a significant gain in test scores from pre-test to T1: nap (11.7 ± 2.0, mean % ± s.e.m., $P < 0.0001$) and non-nap (11.5 ± 2.0, mean % ± s.e.m., $P < 0.0001$). This gain was sustained in the nap group for T2 (13.2 ± 3.1, mean % ± s.e.m., $P < 0.0001$) and T5 (11.1 ± 3.9, mean % ± s.e.m., $P = 0.016$), but in the non-nap group it halved at T2 (7.1 ± 2.8, mean % ± s.e.m., $P = 0.039$) and decayed completely at T5 (1.9 ± 3.3, mean % ± s.e.m., $P = 0.568$) in the non-nap group.

A total of 213 students (mean age 11.0 years old) participated in Experiment II. **Figure 3** shows performance 5 days after class (T5) in the three conditions: nap, non-nap with regular English class, and non-nap with game class in English. Performance was calculated as the ratio between the number of correct answers and the total number of questions in the test. All the three groups in Experiment II displayed a significant decrease in test scores after 5 days ($P = 0.006$): 71.7 ± 2.2 (mean % ± s.e.m.) for the nap group; 66.8 ± 2.3 (mean % ± s.e.m.) for the game class group; and 65.3 ± 2.0 (mean % ± s.e.m.) for the regular class group. A significant difference was found exclusively among the nap and regular class groups ($P = 0.044$); however, T0 already indicated a trend toward this difference between the two groups ($P = 0.055$).

Figure 4 displays the performance of the groups assessed at T0 and T30. As expected, the three groups displayed a significant decrease in test performance after 30 days ($P < 0.0001$): 59.1 ± 1.9 (mean % ± s.e.m.) for the nap group; 54.3 ± 2.2 (mean % ± s.e.m.) for the game class group; and 53.5 ± 2.4 (mean % ± s.e.m.) for the regular class group. No significant differences were found between the groups within each test day (T0 and T30). However, T30 scores revealed a statistical trend for higher performance in the nap group, in comparison with the non-nap class group ($P = 0.090$).

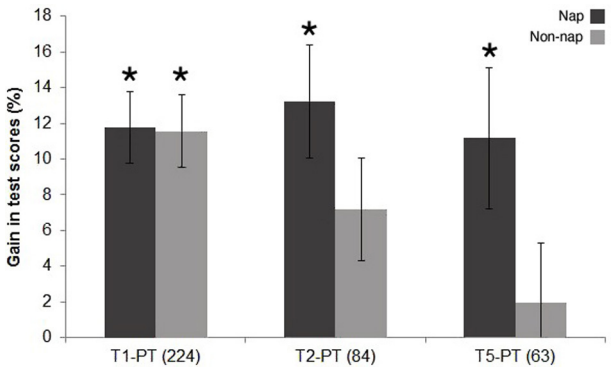

FIGURE 2 | Experiment I—Gains in tests scores between pre- and post-tests applied at 1, 2, or 5 days after learning. Nap ($N = 191$, black bars) and non-nap ($N = 180$, gray bars) groups. Numbers in parenthesis indicate N assessed at each post-test day. Asterisk for $P < 0.05$.

Figure 5 shows the deterioration in performance, from T0 to T5 or T30. At T5, we found a decrease of 9.3 ± 2.3 (mean % ± s.e.m.) for the nap group; 9.0 ± 2.3 (mean % ± s.e.m.) for the game class group; and 9.1 ± 2.6 (mean % ± s.e.m.) for the regular class group. At T30, we found a decrease of 15.2 ± 2.7 (mean % ± s.e.m.) for the nap group; 14.8 ± 2.4 (mean % ± s.e.m.) for the game class group; and 19.2 ± 2.4 (mean % ± s.e.m.) for the regular class group. A two-way ANOVA detected a significant effect of time ($P = 0.0006$) but no significant difference across the T0, T5 and T30 groups ($P = 0.6296$), nor a significant interaction between time and groups ($P = 0.6193$). At T110, no group showed significant differences from chance

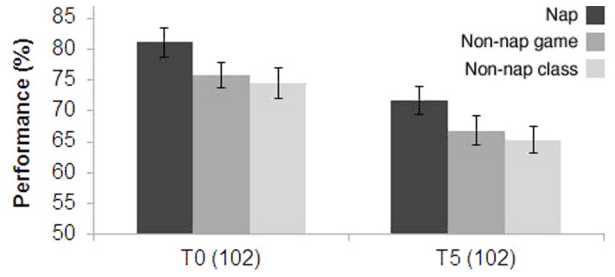

FIGURE 3 | Experiment II—Test performance immediately after the lecture (T0), and 5 days later (T5). Nap ($N = 32$), non-nap groups for game class ($N = 41$), and non-nap groups for regular class ($N = 29$).

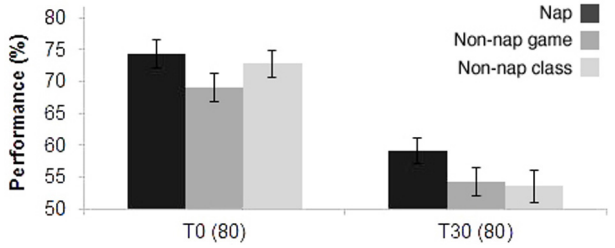

FIGURE 4 | Experiment II—Test performance immediately after the lecture (T0), and 30 days later (T30). Nap ($N = 23$), non-nap groups for game class ($N = 30$), and non-nap groups for regular class ($N = 27$).

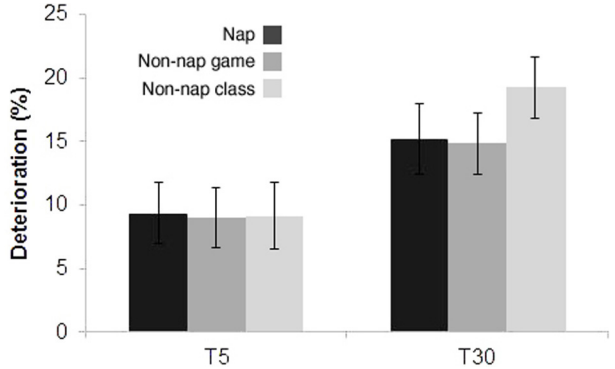

FIGURE 5 | Experiment II—Learning deterioration over time. Difference in performance, from T0 to T5 or T30.

performance ($N = 31$ per group). Across all groups, there were no significant differences in the time that students reported to have slept in the night prior to the tests.

DISCUSSION

We investigated whether daytime naps can benefit declarative learning by adolescents in the school setting. The results of Experiment I suggest that the benefits of a nap taken immediately after school learning remain stable for at least 5 days after the initial learning. These benefits cannot be attributed to putative attention deficits due to less sleep in the non-nap group, since the surprise tests were always applied after at least one full night of sleep. Therefore, the recently acquired memories must have been positively impacted by the nap itself. The results suggest that the lecture-related memories in the non-nap group were more fragile than in the nap group, and a full night of sleep many hours after learning was not enough to compensate for such fragility. The results in Experiment II were inconclusive, because the differences between the nap and non-nap groups were already present at T0, and did not change in proportion over time. While the results of Experiment I indicate that daytime nap can be used to enhance school learning, the results in Experiment II demand a better controlled replication.

Experiments I and II were not designed to test the effect of a single variable of interest, but rather represented two separate attempts to assess the cognitive potential of naps in the school setting, under quite different constraints. A major difference between the two experiments was the length of nap allowed. While Experiment I targeted a 2 h interval, which provides ample opportunity for napping and even for traversing a complete sleep-wake cycle, Experiment II conformed to the standard 50 min per class that is the norm in Brazilian schools. Therefore, differences in nap length may well account for the differences in the results obtained from the two experiments. In that respect, an important limitation of our study is the fact that the students were not recorded with an actimeter or an electroencephalograph, which would have provided quantitative information about sleep amount and the relative contribution of different sleep stages.

In Experiment I, baseline (pre-lecture) knowledge was measured and then each student was subjected to a surprise post-lecture test with highly overlapping content but shuffled options for multiple responses (Supplementary Materials 1, 2). The rationale for this testing scheme was to avoid habituation to the same stimuli, mimicking a pedagogic procedure often employed by teachers to assess learning over time. The lack of testing immediately after the lecture aimed to avoid a "testing effect" prior to consolidation (Carrier and Pashler, 1992; Roediger and Karpicke, 2006). The design of Experiment II, in contrast with Experiment I, assessed exactly the same information immediately post-lecture and again after 5, 30 or 110 days, allowing for a direct measurement of memory retention. Pre-testing was not performed in this case to avoid a ceiling effect at T0. Neither experiment measured knowledge acquisition specifically across the learning experience, making it impossible to determine whether new learning occurred or not during the lecture. Yet, both designs assessed the relationship between post-lecture sleep and memory retention, with reference to pre- and post-lecture levels, for designs I and II respectively. Experiments I and II differed by two variables with respect to testing, and additional experiments would be necessary to determine the effects of testing immediately before or after the lecture, an aspect beyond the scope of this work. Since 2009 we initiated several experiments related to those described in this study, but most were botched due to the frequent occurrence of teacher strikes in Brazilian schools. In Experiment II, the group assessed after 110 days was originally intended to be tested after 30 days, but a strike intervened. In that sense, the design differences in Experiments I and II, without additional experiments able to separately test all the variables involved, stem from the tentative dynamics of classroom research in Brazil. The

difficulties we faced likely apply to classroom research in most of the developing world.

Data were collected both in the morning and in the afternoon for practical reasons: In the Brazilian state of Rio Grande do Norte, where the study was carried out, some schools offer 6th grade classes in the morning but not in the afternoon and vice-versa, because students stay in school only half a day. Although this introduced an additional variable in the study, we found no statistical interaction ($P = 0.18$) between time (morning and afternoon) and condition (nap vs. no nap), in line with previously published data showing equivalent sleepiness at 08:00 and 14:00 among Rio Grande do Norte adolescent students during school days (Sousa et al., 2009). For this reasons, morning and afternoon data were pooled.

It is important to note that sleep is also involved in forgetting (Rolls et al., 2013; Atherton et al., 2014; Oudiette et al., 2014). The present study was specifically aimed at assessing the role of sleep in memory retention, but it was not designed to address forgetting. Studies which set out to investigate the role of sleep in forgetting usually have a distinct paradigm, i.e., they often cue participants in the encoding phase either to remember or forget specific information after sleep. For instance, Saletin et al. (2011) demonstrated that sleep, relative to time spent in waking, can selectively enhance the recall for words previously cued for remembering. In contrast, no such facilitation was observed for items tagged for forgetting. In our study the participants were not cued for remembering nor forgetting, since they were not aware of the post-learning test. Therefore, the issue of forgetting could not be directly addressed by our research design.

Social jetlag plays a key role in the health and functioning of adolescents. Delaying the time of school onset for adolescents and allowing them to nap between classes are relatively easy implementations with potential to reduce classroom sleepiness in a cost-effective manner, thus representing "low-hanging fruits" in the application of neuroscience findings to school education (Ribeiro and Stickgold, 2014; Sigman et al., 2014). School learning is a complex cognitive endeavor, influenced by a myriad of factors beyond physiology, such as the teachers' motivation to teach, the students' intrinsic and extrinsic motivations to learn; the teacher's level of education; the degree of family support etc. Despite all this complexity, the experiments reported here support the use of daytime naps to enhance the duration of declarative contents learned in school. One problem that needs to be addressed is that the use of naps to benefit school learning would likely decrease the time spent in classes undergoing formal exposure to novel and old contents. To prevent a negative impact on school curriculum, the time and amount of post-learning naps must be optimized.

AUTHOR CONTRIBUTIONS

Sidarta Ribeiro and Nathalia Lemos conceived and designed the experiments; Nathalia Lemos, Janaina Weissheimer and Sidarta Ribeiro performed the experiments; Nathalia Lemos and Sidarta Ribeiro analyzed the data; Sidarta Ribeiro, Nathalia Lemos and Janaina Weissheimer wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fnsys.2014.00103/abstract

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Enhancement of sleep slow waves: underlying mechanisms and practical consequences

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Even modest sleep restriction, especially the loss of sleep slow wave activity (SWA), is invariably associated with slower electroencephalogram (EEG) activity during wake, the occurrence of local sleep in an otherwise awake brain, and impaired performance due to cognitive and memory deficits. Recent studies not only confirm the beneficial role of sleep in memory consolidation, but also point to a specific role for sleep slow waves. Thus, the implementation of methods to enhance sleep slow waves without unwanted arousals or lightening of sleep could have significant practical implications. Here we first review the evidence that it is possible to enhance sleep slow waves in humans using transcranial direct-current stimulation (tDCS) and transcranial magnetic stimulation. Since these methods are currently impractical and their safety is questionable, especially for chronic long-term exposure, we then discuss novel data suggesting that it is possible to enhance slow waves using sensory stimuli. We consider the physiology of the K-complex (KC), a peripheral evoked slow wave, and show that, among different sensory modalities, acoustic stimulation is the most effective in increasing the magnitude of slow waves, likely through the activation of non-lemniscal ascending pathways to the thalamo-cortical system. In addition, we discuss how intensity and frequency of the acoustic stimuli, as well as exact timing and pattern of stimulation, affect sleep enhancement. Finally, we discuss automated algorithms that read the EEG and, in real-time, adjust the stimulation parameters in a closed-loop manner to obtain an increase in sleep slow waves and avoid undesirable arousals. In conclusion, while discussing the mechanisms that underlie the generation of sleep slow waves, we review the converging evidence showing that acoustic stimulation is safe and represents an ideal tool for slow wave sleep (SWS) enhancement.

Keywords: EEG, acoustic stimulation, arousal systems, closed-loop, NREM sleep

Sleep is thought to be a universal phenomenon. Despite representing a behavioral state of almost total disconnection from the environment and, therefore, being inherently dangerous, sleep has been identified in every species carefully studied so far (Tobler, 2005; Cirelli and Tononi, 2008; Tononi and Cirelli, 2012). It is unknown when and why sleep emerged in evolution, but the simplest hypothesis is that sleep evolved to serve at least one core function in all species (Cirelli and Tononi, 2008).

Sleep is a tightly regulated homeostatic process. A sleep deficit elicits a compensatory increase in the intensity and duration of sleep, while excessive sleep reduces sleep propensity (Borbély and Achermann, 1999, 2000, 2005; Cirelli and Tononi, 2008). In addition, the quality of wake impacts the intensity of subsequent sleep; in both rodents and humans, intensive learning involving a specific brain region induced a local increase of slow wave activity (SWA, the EEG power between 0.5 and 4 Hz during non-rapid eye movement, NREM sleep) in the very same region during the following sleep, suggesting an experience-dependent local regulation of sleep (Huber et al., 2004, 2006; Vyazovskiy and Tobler, 2008; Hanlon et al., 2009; Krueger and Tononi, 2011; Hung et al., 2013).

It has been proposed that sleep is needed to reestablish "synaptic homeostasis", which is challenged by the remarkable plasticity of the brain (Tononi and Cirelli, 2003, 2006, 2014). Plastic processes occurring during wakefulness result in a net increase in synaptic strength in many brain circuits. Increased synaptic strength has various costs at the cellular and systems level including higher energy consumption (Attwell and Laughlin, 2001), greater need of cellular supplies to synapses leading to cellular stress (Kuhl et al., 1992; Li et al., 2004), and associated changes in glial cells (Reichenbach et al., 2010). Increased synaptic strength also reduces the selectivity of neuronal responses (Balduzzi and Tononi, 2013) and saturates the ability to learn, as suggested by electrophysiological evidence in neocortex and hippocampus (Foster et al., 1996; Rioult-Pedotti et al., 1998, 2000; Whitlock et al., 2006). By renormalizing synaptic strength, sleep could therefore restore the brain to a baseline condition

(Tononi and Cirelli, 2003, 2006, 2014). Several lines of evidence have also shown that lack of sleep leads unavoidably to negative consequences for the organism (Montagna and Lugaresi, 2002; Rechtschaffen and Bergmann, 2002; Shaw et al., 2002; Patel and Hu, 2008; Van Cauter et al., 2008). In humans, even modest sleep restriction leads to cognitive impairment, decreased work productivity, mortality related to automobile crashes, and other adverse events likely related to intrusion of sleep into waking (Banks and Dinges, 2007). These short-lasting sleep-like events, known as behavioral microsleeps, can manifest as a complete failure to respond during an active task, slow eye movements, cessation of blinking and/or head nods, and are usually accompanied by an increase in theta activity in the waking electroencephalogram (EEG; Priest et al., 2001; Blaivas et al., 2007). In addition to "global" microsleeps, sleep deprivation can lead to the occurrence of local sleep-like activity (OFF periods in neuronal firing, typically associated with a local theta or slow wave) in an otherwise awake brain, and cause specific, intermittent performance impairments (Vyazovskiy et al., 2011). This "local sleep" phenomenon was initially demonstrated in rats, but it was recently described also in humans (Hung et al., 2013).

SLOW WAVES AND THEIR IMPORTANCE IN SLEEP

The best characterized marker of the homeostatic regulation of sleep are the slow waves of NREM sleep (Achermann and Borbély, 2003). They are the most prominent EEG event during sleep; they appear as spontaneous large oscillations of the EEG signal occurring approximately once every second in the deepest stage of NREM sleep. Each oscillation consists of an up state, in which neurons fire irregularly at frequencies typical of waking or higher, followed by a hyperpolarized phase, where neurons cease to fire (down state) (Steriade et al., 1993b, 2001; Amzica and Steriade, 1998b; Destexhe et al., 1999).

A compelling feature of slow waves is that they are homeostatically regulated. In general, the longer one has been awake, the more frequent and larger are the slow waves during the subsequent sleep. Therefore, SWA is used as an index of sleep need. Sleep SWA is high in early sleep, when sleep pressure is physiologically elevated, and decreases progressively to reach low levels in late sleep (Tobler and Borbély, 1986; Franken et al., 1991; Vyazovskiy et al., 2007). Moreover, sleep SWA increases further after sleep deprivation, and is reduced by naps (Borbély and Achermann, 2000; Tobler, 2005).

Recently, it has been proposed that, in adults, the homeostatic decline of SWA during sleep is due to a progressive decrease in synaptic strength, which is thought to increase during wakefulness and decrease during sleep (Tononi and Cirelli, 2003, 2006, 2014). There is also some evidence that slow waves may reflect not only the regulation of synaptic strength, but also have a direct causal role in mediating synaptic renormalization during sleep. Experiments performed in rodents and computer simulations have shown that the alternate periods of discharge and silence of neurons, which characterize the behavior of thalamo-cortical neurons during the slow waves, are ideally suited to induce synaptic depression (Kemp and Bashir, 2001; Lubenov and Siapas, 2008; Lanté et al., 2011). In addition, the low levels of acetylcholine and catecholamines that exist during slow wave sleep (SWS) may facilitate the occurrence of synaptic depression (Harley, 1991; Seol et al., 2007).

According to this view, slow waves should mediate at least some of the beneficial functions of sleep on the brain (Tononi and Cirelli, 2003, 2006, 2014). Indeed, several lines of evidence support this prediction. The role of slow waves in the consolidation of memories has been investigated extensively (Marshall and Born, 2007; Diekelmann and Born, 2010; Rasch and Born, 2013). For example, when assessing post-learning changes in sleep, a local increase of SWA was observed in the very same brain areas previously activated when subjects learned implicitly to adapt their movements to a rotated display (Huber et al., 2004). Moreover, the local increase in SWA after learning correlated with improved performance in the rotation adaptation task after sleep (Huber et al., 2004). By contrast, 12 h of arm immobilization induced a reduction of performance in a motor reaching task, and a subsequent decrease of SWA during sleep over the brain regions involved in that task, thereby confirming the tight link between slow waves and learning (Huber et al., 2006). Along the same lines, it was also reported that slow waves (<1 Hz) are more synchronized following intense declarative learning (Mölle et al., 2004). Other studies demonstrated better retention of declarative memories after SWS than after a control interval filled with wakefulness (Plihal and Born, 1997), and the improvement was greater with longer sleep duration (Diekelmann et al., 2012). In addition, Wilhelm et al. (2011) found that memories expected to be retrieved were the ones that benefited the most from SWS. Perhaps the most direct demonstration about the beneficial role of slow waves comes from studies in which selective slow wave deprivation during the night was carried out in healthy subjects. This manipulation, which did not affect sleep time and efficiency, prevented the improvement in performance after visuo-motor and visuo-perceptual tasks, and the changes in performance after slow wave deprivation were correlated with SWA changes, suggesting a casual role for slow waves in the sleep-dependent improvement of cognitive performance (Aeschbach et al., 2008; Landsness et al., 2009).

In addition, other studies have reported that the beneficial role of SWS might not be limited to the brain. For instance, there is evidence that both reduction in total sleep duration with SWS largely preserved and marked reduction of SWS with preservation of total sleep duration have a negative impact on the hypothalamic-pituitary-adrenal axis, in particular on the control of glucose metabolism (Van Cauter et al., 2008; Copinschi et al., 2014). Modifications of the cortisol 24 h profile were observed after few days of sleep restriction (Guyon et al., 2014), while a marked reduction of insulin sensitivity was reported after selective suppression of SWS (Tasali et al., 2008). Similarly, the autonomic control of heart rate and body temperature can be affected by sleep loss (Vaara et al., 2009; Romeijn et al., 2012). Although the role of REM or light NREM sleep cannot be easily ruled out in these studies, it is plausible that SWS exerts a beneficial function also on peripheral systems.

SLOW WAVE ENHANCEMENT: A POSSIBLE WAY TO IMPROVE THE RESTORATIVE FUNCTIONS OF SLEEP

Given the pivotal role of slow waves during sleep, it is not surprising that several efforts have been made to increase sleep efficacy by potentiating SWA. Recently, a number of drugs have been shown to increase SWS. Although acting on different synaptic sites, overall the slow wave enhancing effect of these drugs is mediated by enhancing GABAergic transmission. Specifically, clinical investigations showed that both tiagabine and gaboxadol increased the duration of SWS after sleep restriction (Mathias et al., 2001; Walsh et al., 2008; Walsh, 2009; Feld et al., 2013). Tiagabine also improved performance on cognitive tasks evaluating executive functions and reduced the negative effects of sleep restriction on alertness (Walsh et al., 2006). Although these results are promising, pharmacological approaches to sleep enhancement often raise issues related to dependence and tolerance, and are commonly associated with residual daytime side effects.

To overcome these limitations, one strategy is to enhance deep sleep non-pharmacologically, by stimulating the brain with electrical currents or magnetic fields. A study by Marshall et al. (2006) used intermittent transcranial direct-current stimulation (tDCS) applied at 0.75 Hz for 5-min intervals separated by 1-min off periods after SWS onset, and found an increase in the EEG power in the slow oscillation band (<1 Hz) during the stimulation-free intervals. This increase was associated with enhanced retention of hippocampal-dependent declarative memories, suggesting a causal role for slow waves in sleep-associated memory consolidation (Marshall et al., 2006). Using a similar paradigm, Reato et al. (2013) reported an acceleration of the SWA homeostatic decay in subjects stimulated by tDCS at the beginning of SWS. However, the actual impact of tDCS on physiological sleep is hard to evaluate for several reasons. The recorded EEG during the stimulation is strongly affected by electrical artifacts, preventing a detailed EEG analysis. Furthermore, although tDCS results in sustained and widespread changes in regional neuronal activity, it produces a complex pattern of activated and deactivated brain areas, making the impact on slow waves difficult to predict (Lang et al., 2005). In another study, Massimini et al. (2007) demonstrated that slow waves can be triggered by directly perturbing the cortex during NREM sleep using trans-cranial magnetic stimulation (TMS). Unlike tDCS, the EEG could be recorded concurrently to test the direct impact of TMS. Importantly, virtually every TMS pulse, when in the appropriate location, was able to trigger a full-fledged slow wave that started under the coil and spread to the rest of the brain. However, the long-term effect of repeated exposure to either tDCS or TMS is unknown. Thus, other research has focused on the possibility of inducing slow waves in a more physiological natural manner, by exploiting sensory stimulation.

Historically, vestibular stimulation was the first to be tested as a tool to promote sleep induction, perhaps because of the long-standing notion that physical rocking of a baby, or swinging in a hammock, can be helpful in inducing sleep (Woodward et al., 1990). Indeed, studies carried out in infants demonstrated that vestibular stimulation decreased the proportion of active behavior and concomitantly increased the time spent in quiet sleep (Cordero et al., 1986). In a larger study in healthy adults, bilateral electrical stimulation of the vestibular apparatus shortened sleep onset latency in comparison to sham nights where no stimulation was provided (Krystal et al., 2010). Another recent study reported a facilitated transition from waking to sleep and an increase of SWA in subjects exposed to gentle rocking during an afternoon nap (Bayer et al., 2011). According to the authors, at least one of the mechanisms by which vestibular and, more generally, proprioceptive stimulation could promote sleep and boost SWA involves direct or indirect vestibular and somatosensory projections to the reticular formation, thalamus, and hypothalamus, which could in turn enhance synchronous activity in thalamo-cortical networks (Bayer et al., 2011). In rats, olfactory stimulation induces slow waves (Fontanini et al., 2003; Fontanini and Bower, 2006), but this method had no effect when applied to humans (Tononi et al., 2010), probably because olfactory stimuli have only modest impact on human thalamo-cortical networks (Carskadon and Herz, 2004).

The effect of somatosensory and auditory stimulation was assessed by Tononi et al. (2010). While the change observed with somatosensory stimulation was minor, acoustic stimulation was particularly efficacious in enhancing sleep slow waves. Specifically, using an intermittent stimulation in which tones were played in blocks of 15 s spaced out by stimulation-free intervals, slow waves appeared remarkably large and numerous during the stimulation blocks (**Figure 1A**; Riedner et al., 2012). Thus, when compared to the temporally adjacent stimulation free intervals, stimulation blocks displayed increases in SWA (ranging from 6 to 27%), whereas band-limited power (BLP) in the alpha (8–12 Hz), spindle (12–16 Hz), and beta (16–25 Hz) ranges did not change significantly (**Figure 1B**). In addition, high-density EEG studies (hdEEG, 256 channels) showed that the morphology, topography, and travelling patterns of induced slow waves were indistinguishable from those of spontaneous slow waves observed during natural sleep (**Figure 1C**). A recent study designed to establish the capacity to learn during sleep used acoustic tones delivered during NREM and REM sleep, and found that EEG SWA increased following tone presentation during NREM sleep (Arzi et al., 2012). In another recent study healthy young subjects were exposed to continuous acoustic stimulation at 0.8 Hz starting 2 min before lights were turned off and lasting for 90 min. Subsequent staging and EEG analysis showed an increase of slow oscillation activity (0.5–1 Hz) during the rhythmic stimulation as compared to a sham condition with no stimulation (Ngo et al., 2013a). Of note, the stimulation did not increase the number of total arousals, despite the fact that subjects took longer to fall asleep when stimulated (Ngo et al., 2013a). Lastly, the effectiveness of acoustic stimulation in enhancing slow waves was confirmed in another study in which acoustic pulses delivered during the slow wave up states increased the size of the following slow waves (<1 Hz). The stimulation also improved declarative memory performance as compared to control nights when either the stimulation was delivered out of phase (during the down states) or no stimulation was provided (Ngo et al., 2013b).

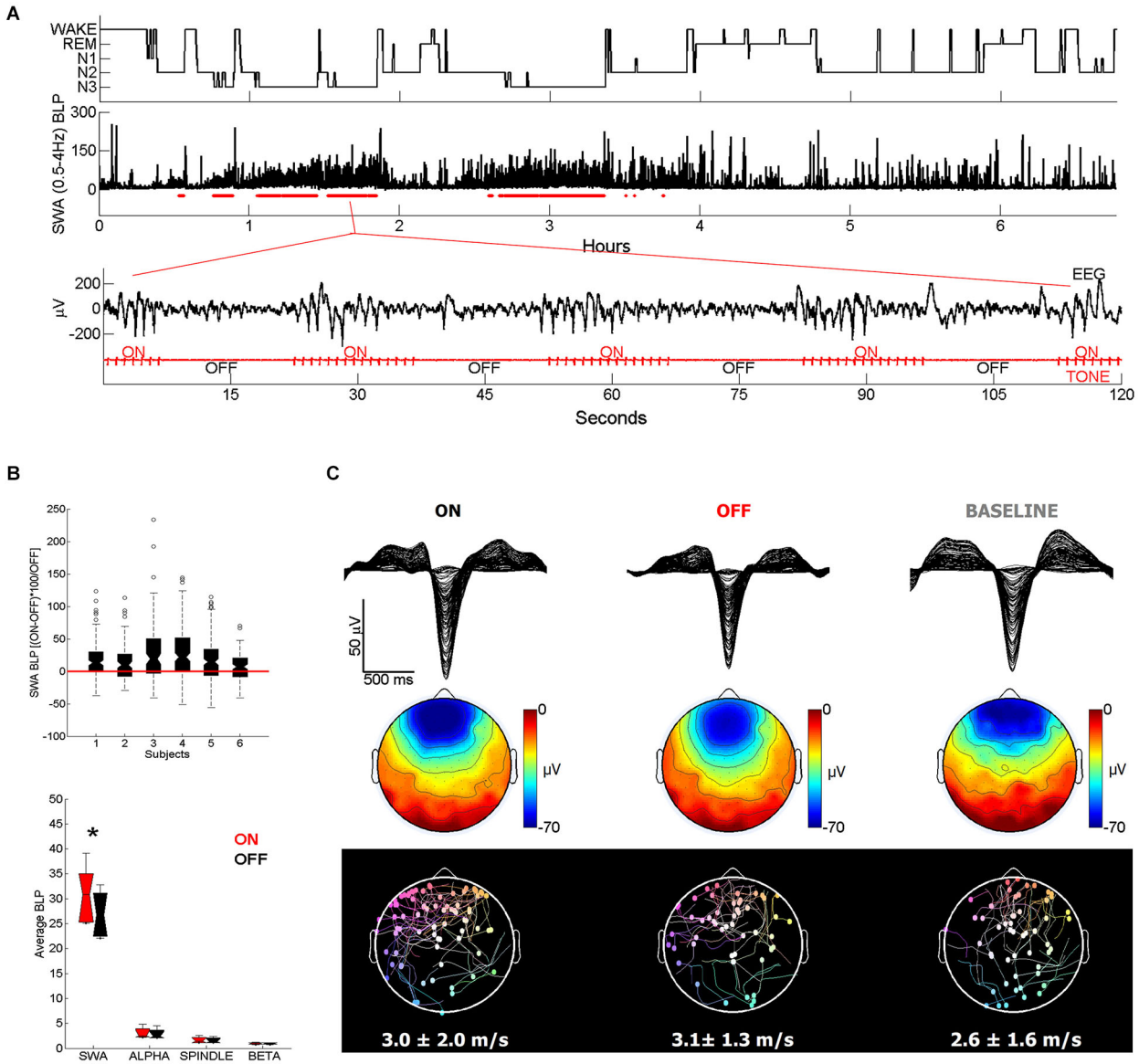

FIGURE 1 | (A) Representative example of acoustic stimulation delivered in 15-s blocks during deeper stages of NREM sleep (N2 and N3, 50 ms tones played with an inter-tone-interval of 1 s). A custom algorithm delivered acoustic stimuli automatically, using the ongoing EEG to examine sleep and adjust tone timing and volume. Hypnogram and SWA band-limited power (BLP) for channel F3-M2 along with the stimulation blocks shown below in red (2 min of EEG and tone stimulation are expanded below). Note that during ON blocks slow waves are more numerous and larger. **(B)** All subjects (n = 6) showed overall increases in SWA (top plot) in ON blocks relative to the temporally adjacent OFF blocks, while other frequency ranges did not change (bottom plot). * indicates significantly different based on a paired t-test, Bonferroni corrected for multiple comparisons ($p < 0.0125$). **(C)** Top plot shows a butterfly plot (all channels overlaid) averaging across all 100 slow waves aligned by the negative peak. Slow waves were randomly selected for comparison from the ON and OFF periods of the stimulation night and from a BASELINE (no stimulation) night. Middle plot shows the average scalp voltage topography at the negative peak. Bottom plot shows the traveling of individual waves and their average speed below. Each dot represents the origin of the wave and the line describes its traveling. Slow waves were detected globally based on standard criteria and traveling was calculated from the negative peak lag distribution of each wave (Siclari et al., 2014).

In conclusion, although other modalities of sensory stimulation (e.g., vestibular) deserve consideration, there is converging evidence that auditory stimulation is a good choice for enhancing slow waves, because it is safe, easily controllable, and can be administered non-obtrusively during sleep.

ENHANCING SLOW WAVES WITH ACOUSTIC STIMULATION

The use of acoustic stimulation in sleep and EEG research is almost as old as the fields themselves. In sleep research, acoustic stimuli are commonly used to test the change in arousal threshold as a function of sleep depth. Further studies assessed

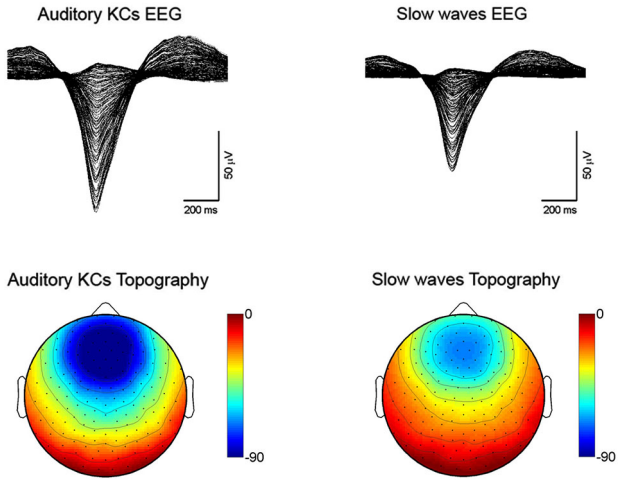

FIGURE 2 | Sleep recordings performed with a HydroCel 256 channel hdEEG net (Electrical Geodesics Inc.) using NetStation software in healthy subjects ($n = 5$). Auditory tones were delivered through speakers or headphones during N2-N3 sleep stages. Auditory KCs and slow waves were detected globally based on period-amplitude criteria (peak to peak minimum amplitude: 75 μV; negative going zero-crossing to positive going zero-crossings greater than 250 ms and less than 1000 ms). For the KCs, an additional criterion was that the waves had to occur between 200 and 1100 ms after the auditory stimulus. Top figures are butterfly plots (all channels overlaid) averaging across all auditory evoked KCs (on the left) and all spontaneous slow waves (on the right). Both waves were aligned by the negative peak. Bottom plots show the average scalp voltage topography at the negative peak for KCs (on the left) and spontaneous slow waves (on the right).

the different brain responses to auditory stimulation across the sleep/wake cycle, and others investigated the role of rhythmic stimulation on the acceleration of sleep onset (Grossman, 1949; Oswald, 1960; Zung and Wilson, 1961; Bohlin, 1971). Nonetheless, how acoustic stimulation leads to slow wave enhancement is still an open question. In the following section we offer a working hypothesis on the underlying mechanisms after reviewing the physiology of the K-complex (KC), a peripherally evoked slow wave, and some relevant facts about the lemniscal and non-lemniscal pathways ascending to the cerebral cortex.

KCs AND SLOW WAVES

The KC was first described by Loomis in 1937 as a distinctive, large wave occurring during light stages of NREM sleep, often in response to sensory stimuli, and consisting of a brief negative peak of several hundreds microvolts followed by a slower positive component (Loomis et al., 1938). According to this definition, both KCs and spontaneous slow waves are "delta" waves occurring during NREM sleep (Colrain, 2005). Indeed, they both are characterized by a large and sharp negative deflection of the EEG line (**Figure 2**, top panels). Moreover, the topographic distribution of this negative component, which represents the biggest portion of the wave, is fronto-central and bilaterally symmetrical for both KCs and slow waves (**Figure 2**, bottom panels).

In addition, there is some evidence suggesting that KCs, like slow waves, might be homeostatically regulated. It has been reported that: (1) KCs density declines from evening to morning and from cycle to cycle across the night of sleep (De Gennaro et al., 2000; Curcio et al., 2003; Sforza et al., 2004; Halász, 2005); (2) KCs are more frequent during a night of recovery sleep following fragmented sleep compared to a baseline night (Nicholas et al., 2002b); (3) KC amplitude tends to be bigger after sleep deprivation, although results are inconsistent (Nicholas et al., 2002b; Peszka and Harsh, 2002; Curcio et al., 2003; Sforza et al., 2004); (4) KCs are smaller and rarer in the elderly and in alcoholics, who also show a large decline of SWA (Nicholas et al., 2002a).

A definitive link between slow waves and KCs was provided by Amzica and Steriade in experiments conducted in cats, which revealed the existence of a slow, cortically-generated oscillation within the thalamo-cortical system (Steriade et al., 1991, 1993b, 2001; Amzica and Steriade, 1998a). The slow oscillation is the fundamental cellular phenomenon that underlies both KCs and slow waves, and manifests as a bistability of the resting membrane potential, which transitions from a depolarized up state, when neurons show sustained firing, to a hyperpolarized down state, characterized by neuronal silence (Steriade et al., 1993b, 2001; Amzica and Steriade, 1998b; Destexhe et al., 1999). The mechanisms that trigger and terminate up and down states remain unclear, but it is known that depolarization-dependent K+ currents play a major role (McCormick et al., 1993; Steriade et al., 1993a; Sanchez-Vives and Mccormick, 2000; Timofeev et al., 2000b; Hill and Tononi, 2005), and it is now clear that layer V pyramidal neurons are especially critical for the regulation of up and down state dynamics (Beltramo et al., 2013). There is a close temporal relationship between these cellular phenomena and simultaneously recorded slow waves or KCs: the surface negativity in the EEG signal (or depth positivity in the local field potential, LFP) corresponds to the down state of cortical neurons as recorded intracellularly, and to the suppression of spiking activity as recorded extracellularly, suggesting that EEG or LFP slow waves are a reflection of near-synchronous transitions between up and down states in large populations of cortical neurons (Murata and Kameda, 1963; Calvet et al., 1973; Noda and Adey, 1973; Burns et al., 1979; Steriade et al., 1993c, 2001; Contreras and Steriade, 1995; Mölle et al., 2006; Mukovski et al., 2006; Ji and Wilson, 2007; Luczak et al., 2007; Cash et al., 2009). Therefore, the propensity of neurons to fall inevitably into silent, hyperpolarized states after a period of activation (bistability) underlies the occurrence of both KCs and slow waves during NREM sleep. However, what mechanisms lead specifically to the triggering of KCs or slow waves is still a matter of investigation.

Slow waves have classically been considered spontaneous events, whereas KCs can be triggered by sensory stimulation, most effectively by acoustic stimuli, but also other stimuli, including visceral ones (Davis et al., 1939; Ackner and Pampiglione, 1957; Webster and Colrain, 1998, for a review: Colrain, 2005). However, even with the most efficacious stimulation, not every peripheral stimulus can evoke a KC during NREM sleep. Some authors have suggested that the cerebral cortex may not always be equally receptive during NREM sleep (Prince, 1965; Terzano

et al., 1985; Achermann and Borbély, 1997; Massimini et al., 2003; Vanhatalo et al., 2004; Parrino et al., 2012; Schabus et al., 2012). For example, Vanhatalo et al. (2004) observed a cyclic modulation (at 0.02–0.2 Hz) of cortical excitability in full-band EEG recordings during NREM sleep, reporting a highly synchronized occurrence of KCs with the negative deflection of this infra-slow fluctuation. Earlier animal studies showed that changes in cortical excitability could reflect rhythmic fluctuations of neuronal activity in the midbrain reticular formation (MRF; Roldán and Radil-Weiss, 1970; Oakson and Steriade, 1982, 1983), whereas more recent evidence suggests that these fluctuations can arise from an intrinsic instability of cortical neuronal networks (Sanchez-Vives and Mccormick, 2000; Timofeev et al., 2000a). Another well-known feature is that evoking two KCs in a short interval is virtually impossible because of a refractory period occurring after each KC (Firth, 1973; Bastien and Campbell, 1994). This feature has led to the suggestion that KCs may exert a protective role for cortical arousals (Colrain, 2005; Halász, 2005); specifically, the occurrence of KCs in relation to a potentially arousing stimulus could briefly reduce cortical excitability, the influence of sensory inputs and, thus, preserve sleep continuity.

Among different sensory modalities, auditory stimulation is the most reliable way to induce KCs in humans (Colrain, 2005; Halász, 2005; Riedner et al., 2011). The reason why the thalamocortical system should be particularly sensitive to acoustic stimuli during sleep remains unclear. Since humans are highly visual, one would expect visual stimuli to be at least as effective as acoustic stimuli in inducing KCs. However, unless particularly strong visual stimuli are used (Riedner et al., 2011) this seems not to be the case, probably because acoustic stimuli have a stronger influence on the arousal systems, given the intricate pattern of connections between the acoustic pathways and the reticular formation in the brainstem (Reese et al., 1995a; Yeomans and Frankland, 1995; Cant and Benson, 2003; Hu, 2003). Moreover, in micro-osmatic animals like humans, the auditory system may be the best system capable of monitoring the environment and detecting distant threats during sleep (Velluti, 1997). By contrast, in osmatic animals, like rodents, this sentinel role may be also played by the olfactory system, which may explain why olfactory stimuli are particularly effective in inducing slow waves in rodents (Fontanini et al., 2003; Fontanini and Bower, 2006).

K-complexes are highly stereotypical in shape and topographic distribution, independent of the stimulus used to evoke them. Because of this consistency, they are often viewed as the result of a non-specific diffuse response of the brain to any sensory stimulus (Numminen et al., 1996; Halász, 2005; Riedner et al., 2011). However, this notion has been challenged by recent studies that have provided evidence for the activation of specific primary sensory areas during the induction of KCs, suggesting the existence of a local component in conjunction with the more diffuse activation. A recent study performed source modeling of high-density EEG recordings in NREM sleep during three different kinds of stimulation (auditory, somatosensory, and visual) (Riedner et al., 2011). As expected, similar source topography was observed for the large negative portion of the KC across different stimulations, although the magnitude of the activation appeared largest for the acoustic stimulation. A more fine-grained analysis, however, showed that some aspects of the KC response were not entirely sensory pathway independent (**Figure 3**). Specifically, higher activation of primary visual areas was observed after visual stimulation, while primary somatosensory and motor areas were more activated after somatosensory stimulation. The primary auditory area, instead, showed a similar level of activation during all types of stimulation, including acoustic stimulation. The authors suggested that the lack of a specific relative auditory activation could have been related to the fact that auditory cortex is particularly difficult to image with source modeling, but could also have been related to the fact that this area may be involved in all evoked KCs, regardless of stimulation modality (see below) (Riedner et al., 2011). However, neuroimaging studies have found that during the presentation of the subject's name or following an acoustic tone the auditory cortex is activated to the same extent in wake and NREM sleep (Portas et al., 2000). Another study that combined EEG and fMRI during NREM sleep found that only tones that were able to evoke a KC led to an activation of primary auditory cortex (Czisch et al., 2009; Dang-Vu et al., 2011). Of note, the same study also showed concomitant activation of the frontal midline regions during the tone-evoked KC (Czisch et al., 2009).

Thus, these results confirm that KCs are characterized by (1) a diffuse brain response that is topographically consistent across different sensory stimulation modalities, but more pronounced for tones; and (2) a local response that is sensory specific and involves primary sensory areas. How sensory stimulation can elicit this dual response is still unclear. One possibility is that the diffuse response is induced by an initial activation of the primary sensory areas that expands through cortico-cortical connections. An alternative explanation is that local and diffuse components reflect the parallel activation of specific and nonspecific ascending sensory pathways, respectively.

LEMNISCAL AND NON-LEMNISCAL ASCENDING PATHWAYS

Ascending sensory pathways are anatomically subdivided into two systems across almost every sensory modality: primary (lemniscal) and secondary (non-lemniscal) (Hu, 2003). Lemniscal pathways are reported to carry a high-fidelity, primary-like representation of stimulus features, while the non-lemniscal pathways supply more information about environmental changes (Kraus et al., 1994; Anderson et al., 2009; Anderson and Linden, 2011). Moreover, the non-lemniscal pathways are usually sensitive to multimodal stimuli (Komura et al., 2005) and display rapid habituation to repetitive stimulation (Calford and Aitkin, 1983; Edeline et al., 1999; Hu, 2003).

In the auditory system, the lemniscal pathways transmit auditory inputs ascending from the cochlear nuclei, while the non-lemniscal pathways consist of fibers coming from several regions of the brainstem. These include the dorsal nucleus of the inferior colliculus (IC), the MRF, the nucleus sagulum (Sag), and the spinothalamic tract (ST; Hu, 2003). In addition, cholinergic nuclei of the pontine reticular formation (PRF) that receive direct

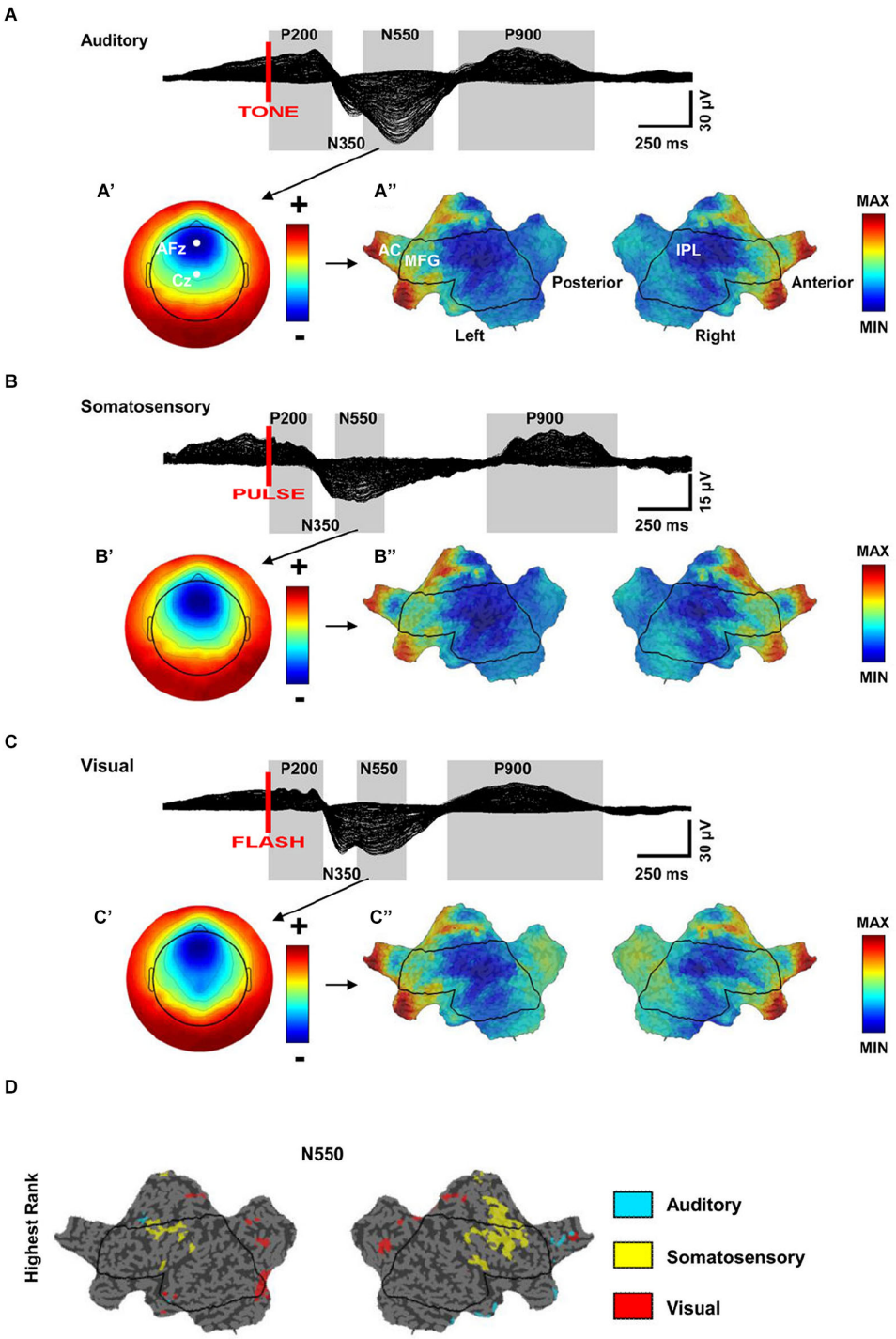

FIGURE 3 | (A–C) Similarity of scalp and source topographies of K-complex (KC) responses. Across subject (n = 7) grand average 256-channel EEG butterfly plot (overlaid traces) of the evoked response during sleep for auditory, somatosensory, and visual stimulation. **(A–C')** Scalp topography for the N550 time periods. Each map is independently scaled in order to indicate relative topography. Red indicates positivity with respect to the average and blue indicates negativity. **(A')** ranges from −30 (blue) to +20 (red); **(B')** ranges from −10 (blue) to +7 (red); **(C')** ranges from −23 (blue) to + 17 (red). **(A–C")** Flat maps of the cortical sources for the N550 peak. Current hot spots (most current) indicated in red, cold spots in blue. AC = Anterior Cingulate, MFG = Middle Frontal Gyrus, IPL = Inferior Parietal Lobule. **(ABC")**, respectively MIN = −1.3, −1.2, −1.4; MAX = 2.6,2.5,2.3. **(D)** Modality-specific differences in cortical sources for the N550 peak of KC. Flat map of significantly different cortical sources across stimulation modalities (Quade test, $p < 0.05$). Color-coding of voxels indicates the stimulation with the highest ranking relative to the other stimulation modalities (adapted from Riedner et al., 2011).

afferents from cochlear nuclei send their contribution to non-lemniscal pathways (Mesulam et al., 1983; Steriade, 1990; Reese et al., 1995b).

In the auditory thalamus (medial geniculate body, MGB) lemniscal and non-lemniscal pathways are clearly segregated and target different neuronal populations. According to Jones's (1998, 2001) hypothesis, thalamic relay cells can be divided in two classes: core and matrix. Core cells receive lemniscal inputs and transmit to primary sensory cortex (layer IV). Matrix cells are targeted by non-lemniscal fibers and diffusely project to associative cortical areas, mainly to layer I. This hypothesis holds for the auditory thalamus (Jones, 1998, 2001; Clascá et al., 2012), where core cells are abundant in the ventral portion of MGB (MGv) that receives lemniscal inputs, whereas matrix cells, targeted by non-lemniscal fibers, prevail in the dorsal and caudo-medial MGB (MGd/MGm) (Jones, 1998; Clascá et al., 2012). It has been proposed that these two regions engage different aspects of auditory function (Hu, 2003). The MGv is tonotopically organized and transmits high-fidelity information to the cortex representing the details of the acoustic environment. The MGd/MGm, instead, projects broadly to the upper layers of associative areas surrounding the primary auditory region, favoring the integration across modalities and across frequencies, displaying delayed responses to acoustic stimuli, and showing rapid habituation to unvarying stimuli (Calford and Aitkin, 1983; King et al., 1995; Miller et al., 2001a,b; He and Hu, 2002; Hu, 2003; Lee and Sherman, 2011). In addition, MGd/MGm seems to have a unique ability to activate the cortex when changes are detected in an otherwise monotonous series of acoustic stimuli (Kraus et al., 1994).

The nucleus gigantocellularis (NGC), in the upper part of the medullary reticular formation, also receives auditory inputs (Martin et al., 2010; Pfaff et al., 2012). This nucleus is connected with several brainstem structures, and plays a major role in activating neurons of the locus coeruleus (LC), the major source of noradrenaline in the brain (Aston-Jones et al., 1986; Van Bockstaele and Aston-Jones, 1995; Berridge and Waterhouse, 2003). In contrast to brainstem glutamatergic and cholinergic ascending projections, which mainly innervate sub-cortical structures, LC neurons innervate directly all cortical layers (Jones and Moore, 1977; Jones et al., 1977; Jones and Yang, 1985; Jones, 2003), fire maximally in response to novel stimuli (Aston-Jones et al., 1996), and are highly effective in activating the cerebral cortex (Berridge and Foote, 1991; Carter et al., 2010; Constantinople and Bruno, 2011). Interestingly, LC fibers and other ascending fibers of the non-lemniscal pathways show rapid habituation to repeated presentation of the same acoustic stimulus (Hervé-Minvielle and Sara, 1995). Overall, there is substantial anatomical overlap between the auditory non-lemniscal pathways and the arousal promoting systems that diffusely project to the thalamo-cortical system, including LC fibers and pontine cholinergic projections (Hu, 2003; Jones, 2003). Since these arousal systems can be broadly activated by acoustic stimulation, we include their ascending projections as part of the "non-lemniscal" pathways (**Figure 4**).

SLOW WAVE ENHANCEMENT: A HYPOTHETICAL MECHANISM

We have seen that non-lemniscal pathways include arousal-promoting neuromodulatory systems with diffuse thalamic and/or cortical projections, but also thalamic matrix cells, which have diffuse cortical projections as well, especially to layer I (Jones, 2001). While the involvement of these projections in activating the thalamo-cortical system during sleep is well established (Jones, 2003; Halász et al., 2004; Riedner et al., 2011), less is known about their role in triggering slow waves in response to a sensory stimulus.

We hypothesize that during NREM sleep auditory inputs capable of activating non-lemniscal pathways can produce near-simultaneous depolarization of many neurons widely distributed over the cortex. Provided that it does not arouse the subject, such "bottom-up" activation may lead to a fast and efficient synchronization of large populations of cortical neurons. Given the bistable behavior of the thalamo-cortical system at this stage of sleep, the rapid and synchronous neuronal depolarization would be inevitably followed by a massive hyperpolarization. At the EEG level, this would lead to an "enhanced" slow wave that displays larger amplitude, steeper slope, and involves broader cortical regions than the majority of spontaneous slow waves, which typically originate independently in many regions of the cortex and involve circumscribed cortical areas (Nir et al., 2011; Siclari et al., 2014). Thus, the net result of the emergence of acoustically induced slow waves during NREM sleep would be an increase in SWA, in line with what has been observed experimentally in several studies (Tononi et al., 2010; Arzi et al., 2012; Ngo et al., 2013a,b, see also **Figure 1**).

SLOW WAVE INDUCTION AND AROUSALS

We have seen that the mechanism by which slow waves can be induced is likely the same one used to arouse the organism when a sudden change indicating a potential danger in the environment is detected. Thus, the intensity of stimulation has to be strong enough to trigger the ascending pathways, but not so strong as to cause a full-blown awakening. This suggests the existence of a threshold below which the stimulation is likely to be completely ineffective, above which the stimulation will be effective, and further above which the stimulation is likely to be disruptive. The idea that the arousal systems can be functionally parceled according to the magnitude of stimulation is not new, and relies on an old concept introduced by Moruzzi in early 1950s. Specifically, he claimed that for mild sensory stimulation only some portions of the activating reticular ascending system (ARAS) might be activated, while the entire system could be recruited only by more intense stimuli (Moruzzi, 1954; Berlucchi, 1997). Although still lacking direct confirmation, there is experimental evidence demonstrating that not all parts of the ARAS are equally effective in arousing the thalamo-cortical system. For example, Constantinople and Bruno (2011) observed that the activation of the noradrenergic, but not cholinergic, pathways were capable of changing the cerebral cortex from a state of slow wave anesthesia to wake. The key role of LC in promoting waking has been confirmed in optogenetic experiments that targeted one arousal system at a

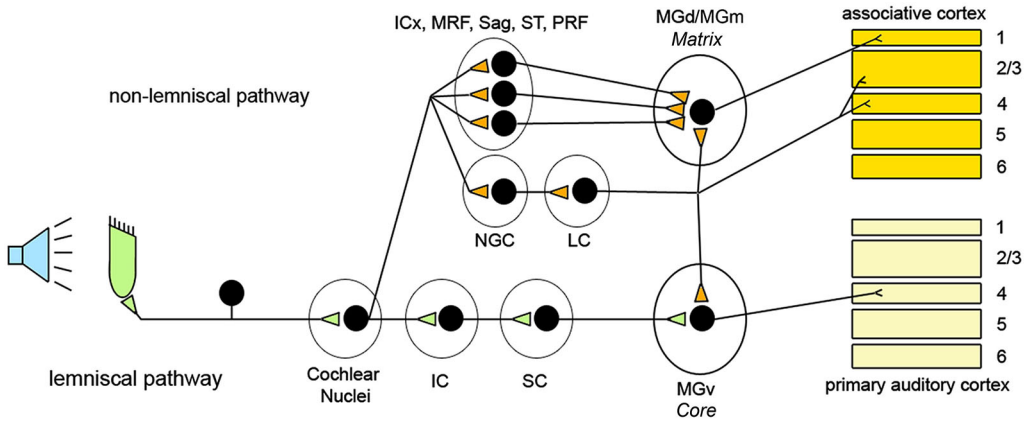

FIGURE 4 | Schematic representation of the organization of the ascending acoustic pathways and relative targets in the thalamus and the cerebral cortex. ICx: shell of the inferior colliculus; MRF: midbrain reticular formation; Sag: Sagulum; ST: spinothalamic tract; PRF: pontine reticular formation; NGC: Nucleus gigantocellularis; LC: Locus coeruleus; IC: Inferior colliculus; SC; Superior colliculus; MGd: Medial geniculate dorsal; MGm: Medial geniculate caudo-medial; MGv: Medial geniculate ventral.

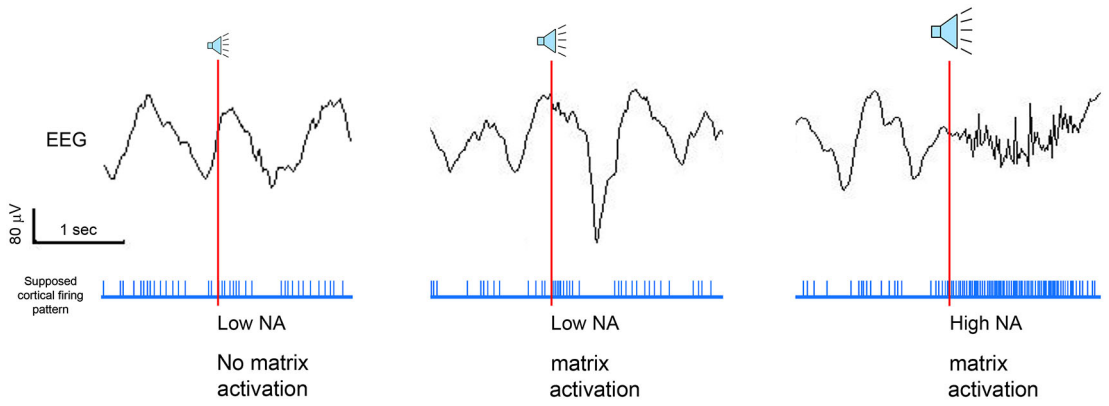

FIGURE 5 | Schematic representation of the hypothetical role of thalamic matrix cells and noradrenaline (NA) in regulating the EEG outcome after tone presentation. We hypothesize that, during NREM sleep, acoustic stimuli can be ineffective, lead to enhanced waves, or provoke cortical arousals, depending on the involvement of the locus coeruleus and/or thalamic matrix cells.

time. In these studies, only the activation of LC caused almost immediate sleep-to-wake transitions from both NREM or REM sleep, suggesting that the LC, more than other arousal pathways, is crucial in promoting wakefulness (Carter et al., 2010; de Lecea et al., 2012). Other systems instead, may either take longer to wake up the subject (e.g., the orexinergic system; Adamantidis et al., 2007), or may be equally effective in promoting the transition from NREM sleep to REM sleep or wake (Han et al., 2014) Therefore, it is reasonable to hypothesize that low-intensity stimuli that induce slow wave enhancement are strong enough to activate several parts of the ARAS, but not the LC (**Figure 5**).

OPTIMIZATION OF ACOUSTIC STIMULATION FOR SLOW WAVE ENHANCEMENT DURING SWS

Having developed a framework for understanding how acoustic stimulation enhances slow waves during sleep, we now consider which features of the stimulation might be most impactful when considering these mechanisms. By reviewing the relevant literature, we point out several features of the acoustic stimulation that may affect the magnitude of the slow wave enhancement. By optimizing these parameters, it should be possible to maximize the effectiveness of the stimulation for increasing slow waves.

1. *Intensity*. The intensity of the acoustic stimulation should be adjusted according to sleep depth in order to avoid undesired arousals and sleep fragmentation. We have previously discussed the existence of a threshold below which the stimulation intensity is effective in enhancing slow waves and above which it causes arousal, and how the activation of the LC can represent the key factor in tuning the balance. However, it is still not clear how this threshold can be defined dynamically throughout the course of sleep and whether there are some features of the EEG signal that can be used to

predict it and adjust the stimulation accordingly. A recent study showed that subjects displaying high spindle density during NREM sleep were more resistant to sleep disruption during auditory stimulation, suggesting that spindle density can be a good indicator of sleep stability (Dang-Vu et al., 2010). Other studies observed that the intrinsic frequency of the spindle changes within a sleep cycle, especially for more frontal spindles (Himanen et al., 2002; Andrillon et al., 2011). Specifically, the frequency of the spindles decreases as sleep deepens and it increases as sleep lightens, describing a U-shape curve within the sleep cycle (Himanen et al., 2002; Andrillon et al., 2011). Since the level of thalamic hyperpolarization dictates the period of spindle oscillations (McCormick and Bal, 1997), it is possible that the frequency of the spindles reflects the level of thalamo-cortical polarization (Andrillon et al., 2011). Thus, the deceleration of spindle frequency reported in deep sleep may indicate an increased hyperpolarization of the thalamo-cortical system during this stage, leading to a stronger resilience to perturbing stimuli. Taken together, these studies suggest that measuring spindle density and frequency can provide important information about the state of the thalamo-cortical system and its sensitivity to external stimuli, and therefore can be used to determine the intensity of the stimulation at any given time during NREM sleep.

2. *Sound frequency*. The sound frequency of the acoustic stimuli should vary randomly for each stimulus to prevent habituation of the non-lemniscal ascending pathways. In contrast to the lemniscal pathways that constantly exhibit high-fidelity responses, non-lemniscal pathways show rapid habituation to trains of identical stimuli (Calford and Aitkin, 1983; King et al., 1995; Miller et al., 2001a,b; He and Hu, 2002; Hu, 2003; Lee and Sherman, 2011). Since the early studies performed in late 1950's by Sokolov (1963) habituation has been characterized as a decreasing pattern of response following repeated sensory stimulation. It was originally thought that habituation was caused by a blockade of inputs to the reticular formation exerted by corticofugal projections (Sokolov, 1963). However, subsequent studies showed that the mechanism underlying habituation resides in the reticular formation itself and is due to a progressive reduction of synaptic efficacy (Groves and Lynch, 1972; Weber et al., 2002; Thompson, 2009). If the function of non-lemniscal pathways is to detect sudden unexpected changes in the environment, then habituation becomes an essential feature to maximize the capability of an organism to sense change in a dynamic environment, and, conversely, to ignore events displaying a repetitive pattern that contain little new useful information. Therefore, when attempting to enhance slow waves it is essential to provide a certain continuous degree of unexpectedness during stimulation, in order to avoid habituation. One possible way to achieve this is suggested by the evidence that, in a sequence of acoustic stimuli, deviant tones showing a sound frequency different from the background elicit an EEG event-related potential called the mismatch negativity in awake subjects (Näätänen et al., 2011). This characteristic response is thought to reflect the brain reaction to changes occurring in a continually updated auditory environment (Winkler et al., 1996; Sussman and Winkler, 2001), and, albeit attenuated in NREM sleep (Chennu and Bekinschtein, 2012), it can be used to diminish habituation and maintain the desirable effect. In practice, a feasible approach would consist of changing sound frequency of every acoustic stimulus in a sequence of stimuli.
3. *Timing*. Ideally, the stimulation should arrive to the cortex during the particular phase of the thalamo-cortical oscillation in which its ability to enhance slow waves is maximal. During SWS, slow waves occur more or less regularly one after another, suggesting that the underlying thalamo-cortical network swings continuously between up and down states. Experimental evidence suggests that the phase of this ongoing oscillation has important consequences for the fate of incoming stimuli. For example, the amplitude of somatosensory evoked potentials tends to increase progressively when the stimulus occurs during the negative slope of the wave (i.e., when thalamo-cortical neurons are becoming hyperpolarized), reaches its maximum at the negative-positive transition, and decays along the positive drift (Massimini et al., 2003). On the other hand, the amplitude of auditory evoked potentials and BOLD responses in the superior temporal gyrus, a higher auditory association cortex, is larger when stimulation occurs just after the negative peak of the EEG slow wave (i.e., when thalamo-cortical neurons are depolarizing) (Schabus et al., 2012). It is hard to evaluate whether either different delays of somatosensory and auditory systems, or inherent delays in the stimulating systems might be responsible for these different results. However, a recent study, using a precise stimulating apparatus with an inherent delay of a few milliseconds, demonstrated that acoustic pulses occurring in correspondence of the slow wave up states were effective in enhancing subsequent slow waves. Conversely, tones delivered immediately after the negative peak of the slow waves had a disruptive effect on the following waves, most likely by interfering with the intrinsic bistable activity of the thalamo-cortical system (Ngo et al., 2013b). These findings are consistent with a previous study performed in rats showing the presence of a refractory period right after the spontaneous EEG down state, when the induction of slow waves was unlikely even under the highest physiological pressure for sleep (Vyazovskiy et al., 2009). Overall, these data indicate that the effect of incoming stimuli is strongly influenced by the phase of the thalamo-cortical oscillation. Thus, the ability of acoustic stimuli to trigger a slow wave relies not only on sufficient activation of the ascending sensory pathways, but is also strongly dependent on the phase of the ongoing oscillation of the thalamo-cortical system.
4. *Entrainment*. In addition to maximizing the efficacy of the response to an individual stimulus, slow wave enhancement can take advantage of the brain's ability to entrain to repetitive stimuli. Entrainment refers to the ability of a periodic external force to synchronize the natural oscillation of a certain system (Pikovsky et al., 2003). Entrainment of EEG brain waves is a well-known phenomenon (Herrmann, 2001; Thut et al., 2011).

The photic driving response was discovered in 1934 by Adrian and Matthews, who first demonstrated the entrainment of the EEG alpha rhythm using intermittent photic stimulation (Adrian and Matthews, 1934). Since then, this technique has been widely used to induce abnormal EEG features in an otherwise normal EEG (Takahashi, 2005). The photic driving response is usually elicited when the stimulation frequency is similar to the intrinsic frequency of the brain oscillations, and it is thought to represent a steady-state resonance response of the brain to periodic stimulations (Walter and Walter, 1949; Mundy-Castle, 1953). Electroencephalogram entrainment to rhythmic sensory stimulation is not limited to the visual modality, and has been reported after somatosensory and auditory stimulation (Pompeiano and Swett, 1962; Rodenburg et al., 1972; Sclabassi et al., 1974). Specifically, slow oscillations can be entrained by rhythmic auditory stimulation during SWS in animals and humans (Gao et al., 2009; Ngo et al., 2013a). The underlying mechanism is not well elucidated, but the intrinsic pacemaker activity of neurons and synaptic connections may give rise to entrainable oscillators (Gao et al., 2009). If so, auditory stimulation could act as a driving force able to organize and synchronize intrinsic brain oscillators. At the EEG level, this would result in waves more organized around the frequency of stimulation, and in increased power associated with that frequency (Ngo et al., 2013a). This view is also in line with the increase of slow oscillation activity (EEG power between 0.5 and 1 Hz) during acoustic stimulation at 0.8 Hz, roughly the spontaneous frequency of the thalamo-cortical system during SWS (Ngo et al., 2013a).

CLOSED-LOOP STIMULATION

We have seen that slow wave enhancement can be optimized by tuning some features of the acoustic stimulation. However, a limiting factor in the ability of enhancing slow waves is that the mechanism responsible for this effect is also the same one used to arouse the cortex from sleep. Playing tones at inappropriate volumes or at inappropriate times during sleep not only can reduce the desirable effect, but can induce arousals, and disrupt sleep. It becomes then imperative to adjust the stimulation parameters moment by moment according to the depth of sleep and, as described above, the brain's changing receptiveness to incoming stimuli.

In practice, such dynamic control can be achieved by developing a system that monitors the EEG and, in real-time, assesses first whether the stimulation can be delivered without the risk of arousing the user, and then adjusts the stimulation properties depending on the ongoing EEG (**Figure 6**). From an implementation perspective the system estimates, with a high sensitivity, the likelihood of an arousal event by considering the EEG power in the higher frequency bands (alpha and beta). In case an arousal is detected during stimulation, the latter should cease. If a sufficiently long period of time (few minutes) has passed since the last arousal event, then the system should attempt to detect (with high specificity) the occurrence of SWS. In case SWS is detected, then the acoustic stimulation should be delivered and its properties adjusted to the ongoing EEG. Specifically, if the slow wave enhancement effect decreases, the intensity of the stimulation increases.

Crucial properties of such real-time system include small inherent delay in acquiring EEG data and high timing accuracy (low jitter) in delivering the stimuli (Hartmann et al., 2011). For example, short delay and narrow jitter would make it possible to target a precise phase of the on-going oscillation or would allow the system to quickly reduce the stimulation intensity when high frequency EEG patterns first appear, heralding an arousal.

ACOUSTIC STIMULATION OUTSIDE NREM SLEEP

There is some evidence that acoustic stimulation could be used not only to enhance NREM slow waves, but also some features of REM sleep (Drucker-Colin et al., 1983; Arankowsky-Sandoval et al., 1986, 1992; Vazquez et al., 1998; Amici et al., 2000). Early studies found an increased number of ponto-geniculo-occipital (PGO) spikes and increased REM duration in animals exposed to auditory stimulation. The authors attributed this effect to the anatomical proximity of the structures involved in acoustic signal processing and REM sleep regulation. More specifically, they suggested that acoustic stimulation could promote the release of acetylcholine in the brainstem structures involved in PGO activity (Arankowsky-Sandoval et al., 1986; Ball et al., 1991). The increased REM duration following acoustic stimulation was later confirmed by another group of researchers showing longer REM periods also for stimulations occurring during NREM sleep (Amici et al., 2000). In humans, an increase in both REM sleep duration and sleep efficiency occurs when acoustic stimulation is started at the beginning of REM sleep, whereas a disruptive effect with a larger number of awakenings has been reported when the stimulation starts near the end of a REM episode (Salin-Pascual et al., 1991). Increased REM sleep duration was also correlated with a pronounced decrease in the density of REMs (Mouze-Amady et al., 1986), and to a better retention of memories in a Morse code learning task (Guerrien et al., 1989). Nevertheless, despite several reports indicating that acoustic stimulation lengthens REM sleep, the behavioral impact of this manipulation requires further investigation.

SUMMARY AND FUTURE DIRECTIONS

Slow waves are the best marker of the homeostatic regulation of sleep, and, most likely, they are responsible for carrying out some physiological functions of sleep for the brain (Tononi and Cirelli, 2003, 2006, 2014). Novel data indicate that it is possible to enhance these slow waves through non-pharmacological means. Initial experiments showed that tDCS and TMS applied to the human cerebral cortex at appropriate frequencies could induce slow waves (Marshall et al., 2006; Massimini et al., 2007). However, these methods are currently impractical and their safety, especially for chronic long-term exposure, is still unknown. Recently, more attention has been given to the possibility of enhancing slow waves by using more physiological stimuli. Among different sensory modalities, acoustic stimulation appears to be the most effective in increasing the magnitude of slow waves. The underlying mechanism is unclear, but we hypothesize that sub-arousal threshold stimuli are capable

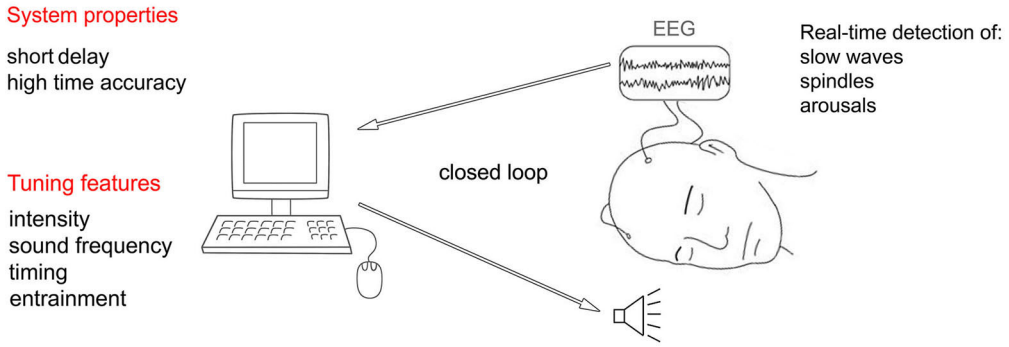

FIGURE 6 | Schematic representation of an automated real-time system capable of adjusting acoustic stimulation parameters according to the ongoing sleep.

of synchronizing the cortical activity of large populations of neurons through the activation of the non-lemniscal pathways that project diffusely over the cerebral cortex. Given the bistable behavior of the thalamo-cortical system during NREM sleep, the rapid and synchronous neuronal depolarization would be inevitably followed by a massive hyperpolarization. At the EEG level, this would result in a slow wave showing large amplitude and steep slope, and involving bilaterally the fronto-central regions.

We then considered which features of the stimulation might be most impactful when considering these mechanisms. By reviewing the relevant literature, we pointed out several features (intensity, sound frequency, timing, and entrainment) of the acoustic stimulation that may play an important role in regulating the efficacy of the stimulation. Specifically, we indicate that stimulation intensity should be tuned according to sleep depth, because there is a threshold below which the stimulation intensity is effective in enhancing slow waves and above which it causes arousal. Changing the sound frequency could counteract the occurrence of habituation and hitting the thalamo-cortical system at convenient times could maximize the enhancing effect. Finally, we argue that repetitive patterns of stimulation can easily entrain endogenous brain rhythms leading to waves more organized around the frequency of stimulation. However, it remains to be determined to what extent these features can be tuned and how they relate to each other. For example, it is possible that hitting the precise phase of the ongoing thalamo-cortical oscillation becomes less important when the brain is entrained by a repetitive stimulation. Moreover, the interaction of these factors may be different in specific subsets of the population, for instance in subjects who are easily aroused during sleep by environmental noise, or in elderly people who typically exhibit reduced amount of SWA. Finally, it would be interesting to assess the potential role of acoustic stimulation during sleep in conditions characterized by decreased SWS, such as chronic sleep restriction and insomnia.

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