CONSEQUENCES OF BRIEF PERIODS OF SLEEP LOSS ON HIPPOCAMPUS-DEPENDENT MEMORY AND SYNAPTIC PLASTICITY

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ABSTRACT

CONSEQUENCES OF BRIEF PERIODS OF SLEEP LOSS ON HIPPOCAMPUS-DEPENDENT MEMORY AND SYNAPTIC PLASTICITY

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Hippocampal cellular and molecular processes critical for memory consolidation are affected by the amount and quality of sleep attained. Questions remain with regard to how sleep enhances memory, what parameters of sleep after learning are optimal for memory consolidation, and what underlying hippocampal molecular players are dysregulated by sleep deprivation to impair memory consolidation and plasticity. In this dissertation, I describe experiments that we performed to identify the time window where memory consolidation is sensitive to sleep loss as well as to characterize two potential molecular players targeted by sleep deprivation. Because consolidation appears to have a particular window where it is sensitive to sleep loss, we explore the parameters of this time window in **Chapter 2**. Our results suggest that a specific 3-hour period of sleep loss during consolidation disrupts both memory and plasticity. In the second portion of this dissertation, I examine the mechanisms by which sleep deprivation impairs hippocampus-dependent memory consolidation. In **Chapter 3**, we show that loss of the phosphodiesterase (PDE) 4A, an enzyme responsible for decreasing cAMP signaling, rescues spatial memory disrupted by sleep loss. These results further implicate cAMP signaling with the negative effects of sleep deprivation on memory. Obtaining adequate sleep is challenging in a society that values "work around the clock." Therefore, the development of interventions to combat the negative cognitive effects of sleep deprivation is critical. However, a limited number of therapeutics exists that are able to enhance cognition in the face of insufficient sleep. The identification of the temporal characteristics of sleep loss and the molecular pathways implicated

in the deleterious effects of sleep deprivation on memory could potentially yield new targets for the development of more effective drugs.

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CHAPTER ONE

GENERAL INTRODUCTION

OVERVIEW

Chronic sleep loss is a widespread problem in our society (Strine and Chapman, 2005). According to the Center for Disease Control, about 7-19% of adults in the US report inadequate sleep, and an estimated 50-70 million Americans suffer from chronic sleep disorders. Insufficient sleep is co-morbid with chronic problems such as heart disease, kidney disease, high blood pressure, diabetes, obesity and mental illness (Ford, 1989; Gillin, 1998; Knutson and Van Cauter, 2008; Hirotsu et al., 2010; Vijayan, 2012; Engeda et al., 2013; Najafian et al., 2013; Palagini et al., 2013). Sleep loss also contributes to irritability, aggression, inattentiveness and diminished psychomotor vigilance (Rajaratnam and Arendt, 2001; Van Dongen et al., 2003; Kamphuis et al., 2012). The negative impact of sleep loss on physical and mental health places a strain on our healthcare system (Kapur et al., 2002) and a large financial burden on our economy (Durmer and Dinges, 2005). Unfortunately, the common myth is that people can function on little sleep with no consequences, even though studies employing both human and animal models demonstrate that mental and physical health requires sufficient sleep (Banks and Dinges, 2007). Because so many people are unable to obtain sufficient sleep on a daily basis, it is critical to understand the molecular and cellular impact of sleep loss in an effort to identify novel therapeutic approaches to counteract these effects.

In the introduction to my thesis, I bridge what is known about critical periods of molecular signaling post-learning with our understanding of the role of sleep and sleep deprivation in memory consolidation. This is followed by my study focusing on the time windows when sleep deprivation can disrupt hippocampal function in **Chapter 2**. I examine 2 molecular targets of sleep deprivation in **Chapters 3** and the **Appendix** (See

Fig. 1 for schematic overview). Finally, I discuss the implications of my findings from these studies in **Chapter 4.**

Section 1. Interplay between sleep and memory

1.1. Molecular signaling consolidates memory

Memory consists of at least 3 stages; encoding, consolidation, and retrieval. Each phase requires specific molecular machinery (Abel and Lattal, 2001). Because the focus of this dissertation is memory consolidation, we elaborate on the molecular signaling underlying this stage of memory processing, termed synaptic consolidation (Dudai, 2004). A large body of work suggests that sleep is particularly beneficial to the consolidation stage of memory storage, and that this stage is vulnerable to sleep manipulation (Sagales and Domino, 1973; Buzsáki, 1998; Stickgold et al., 2000; Graves et al., 2003a). Learning induces a transient increase in calcium (Ca^{2+}) and adenylyl cyclase, an enzyme responsible for production of the second messenger cyclic adenosine monophosphate (cAMP) (Xia and Storm, 2012). cAMP activates 3 downstream targets important for protein synthesis and eventual memory consolidation. These targets include protein kinase A (PKA), exchange protein directly activated by cAMP (Epac) and hyperpolarization-activated cyclic nucleotide-gated channels (HCN channels) (Arnsten, 2007). Activation of these downstream targets, along with neurotrophins and other kinases such as calmodulin dependent protein kinase (CaMKI, CaMKII, and CaMKIV), mitogen activated protein kinase (MAPK), and extracellular signal-regulated kinase (ERK 1/2), leads to phosphorylation of transcription factors (Enslen et al., 1994; Matthews et al., 1994; Roberson et al., 1999; Ahmed and Frey, 2005). Transcription factors such as cAMP response element binding protein (CREB) can be activated by neurotrophin signaling through their receptors (Finkbeiner et al.,

1997) to promote up-regulation of gene expression for proteins that will consolidate labile memories into long-term memories (Roberson and Sweatt, 1999; Roberson et al., 1999). Previous time course studies identified time windows where inhibition of these signaling components in the hippocampus impairs memory consolidation. Two periods for activation of cAMP downstream signaling and protein synthesis, immediately after learning and 4 hours after learning, are required for consolidation (Grecksch and Matthies, 1980; Bourtchouladze et al., 1998). Three peaks in cAMP levels have also been observed after training at 0.5 hours, 3 hours and 6 hours during consolidation (Bernabeu et al., 1997a). Two early sensitive periods during consolidation also exists for mRNA synthesis, ERK1/2 activity, and phosphorylation of CREB (Igaz et al., 2002; Trifilieff et al., 2006). These critical signaling periods appear to occur within the first few hours of sleep post-learning (Graves et al., 2001; Hernandez and Abel, 2011). When sleep loss overlaps with these critical time windows for molecular signaling, consolidation is impaired (Smith and Rose, 1996; Graves et al., 2003a; Palchykova et al., 2006). These sensitive periods of molecular signaling give an approximation of when sleep deprivation could disrupt memory through disruption of molecular signaling processes. I isolate this critical period in which sleep deprivation impairs memory consolidation in **Chapter 2**.

1.2. Distinction between sleep states

To discuss the consequences of sleep or sleep loss on memory, it is crucial to understand the pattern of neuronal electrical activity that occurs during different sleep states. Sleep is measured by polysomnographic recordings that combine electroencephalography (EEG), electro-oculogram (EOG), and electromyography (EMG) to distinguish the stages of sleep (Franken et al., 1991). Sleep cycles between non-rapid

eye movement (NREM) and rapid eye movement (REM) sleep (Stickgold, 1998). In humans, NREM sleep can further be dissected into 4 stages. Rechtshaffen and Kales developed the standard scoring guidelines to determine sleep stage in humans (Rechtschaffen and Kales, 1968). Rolling eye movements are prominent during stage 1 sleep, while slow oscillations, fast sleep spindles (12-15 Hz), and k-complexes are prominent during stage 2. Recordings during stages 3 and 4 of NREM sleep, termed slow wave sleep (SWS), are characterized by high amplitude, low-frequency delta waves (also known as slow waves, 1-4 Hz) and spindle activity (Mölle et al., 2011). NREM sleep is accompanied by decreased muscle tone as assessed by EMG recordings (Steriade et al., 1993a, 1993b). REM sleep EEG exhibits features similar to wake EEG with cortical activation characterized by low voltage fast EEG activity (Hobson and Steriade, 2011). Other common features of REM sleep include complete loss of muscle tone (atonia) (John et al., 2004) and characteristic rapid eye movements detected by EOG (Aserinsky and Kleitman, 1953; Jouvet, 1962). Hippocampal theta rhythms, associated with wake, occur during REM sleep as well (Buzsáki et al., 1983; Greenstein et al., 1988). Interestingly, this oscillatory pattern can be observed in hippocampal and cortical regions during learning in spatial navigation and episodic retrieval (Klemm, 1976; O'Keefe and Burgess, 1999; Klimesch et al., 2001). NREM and REM sleep have been examined in depth in the context of memory to determine the contribution of each stage in the consolidation process.

Sleep patterns differ across species, and this difference needs to be taken into account when reviewing rodent sleep literature. While humans are diurnal and sleep approximately 8 hours/night in a mono-phasic pattern, rodents are nocturnal mammals that exhibit poly-phasic sleep patterns. In humans, a full cycle between NREM and REM sleep takes about 90 minutes with a total of 4-6 cycles per night. Rodents NREM-REM

cycle is much shorter, lasting approximately 10 minutes (Trachsel et al., 1991; Benington et al., 1994). Scoring rules in rodents also differ slightly from humans. While rodents exhibit NREM and REM sleep, NREM can be further broken down to SWS stage I or SWS stage II. SWS I is identified by the presence of sleep spindles in the cortical EEG. SWS II is identified by the presence waves in the range of 0.1-4.0 Hz in the cortical EEG. (Datta and Hobson, 2000; Datta and Maclean, 2007). Despite the differences in human and rodent sleep, the neurobiology regulating sleep/wake states is similar and much has been learned about how sleep affects memory by studying animal models.

1.3. Sleep enhances memory consolidation

Evidence from various human and rodent studies supports the importance of sleep in learning and memory. In 1924, Jenkins and Dallenbach were the first to discover that sleep facilitates long-term memory formation. In this seminal study, human subjects displayed improved memory of nonsense syllables when they slept during the period between learning and recall (Jenkins and Dallenbach, 1924). Since this pivotal discovery termed the ‘sleep effect’, several other research groups have replicated the finding that memory benefits from sleep (Smith, 2001; Born et al., 2006; Gais et al., 2007; Oudiette et al., 2013; Stickgold and Walker, 2013).

Although researchers agree that sleep is more effective than wakefulness for memory consolidation, debates have emerged as to what stage of sleep is beneficial for memory. Initially, most research groups hypothesized that REM sleep enhanced memory consolidation due to its similarity to waking EEG and its increased firing of hippocampal neurons that had been active prior to sleep (Pavlides and Winson, 1989). Much of the support for the role of REM sleep in memory came from animal studies that demonstrated an increase in REM sleep, for brief time windows after learning (Smith,

1985a; Hennevin et al., 1995). The increase in REM sleep after learning has been demonstrated in cats trained in the instrumental learning paradigm (Lecas, 1976). Increases in REM sleep were also observed in rabbits after handling, which led to more activity in successive open field behavior assessment (Denenberg et al., 1977). Rodent studies also showed increases in REM sleep episodes after learning in associative fear related learning tasks, such as the fear conditioning paradigm and an escapable shock task (Sanford et al., 2010; Machida et al., 2013; Menz et al., 2013). REM sleep also increased in rodents after training in the Morris water maze paradigm (Smith and Rose, 1996, 1997). Not only was an increase in REM sleep observed after training, but also REM sleep-related processes such as theta rhythm and pontine-wave activity were identified as important for memory consolidation in rodents trained in the two-way active avoidance task (Datta et al., 2005; Datta and O'Malley, 2013). Many took these observed increases in REM sleep after conditioning as evidence that REM sleep was closely tied to enhancements in the memory process. This hypothesis was supported by rodent studies that involved REM sleep deprivation during these same REM sleep time windows, demonstrating memory impairments following these deprivation periods (Smith, 1985a).

Until the late 1980s, REM sleep was considered to be the critical sleep stage in memory improvement. However, later studies encountered difficulty demonstrating the importance of REM sleep alone in consolidation of memory (Siegel, 2001). Human and rodent studies observed an increase in NREM sleep, and NREM associated processes such as slow wave activity and spindle density after training (Stickgold et al., 2001; Gais et al., 2002; Huber et al., 2004; Hellman and Abel, 2007). A few studies also demonstrated a NREM sleep-memory link in rodents through the discovery of neuronal reactivation, termed 'replay', where the same set of hippocampal neurons previously

active during spatial learning fired in the same sequence during hippocampal sharp-wave ripple events (SPW-R) that occur in NREM SWS (Wilson and McNaughton, 1994; Lee and Wilson, 2002; Ji and Wilson, 2007; Ego-Stengel and Wilson, 2010; Bendor and Wilson, 2012). The role of NREM sleep in memory consolidation was further cemented by Rasch and colleagues who conducted a study where human subjects performed an associative task consisting of card locations paired with a particular odor. The researchers introduced these same smells during SWS, which activated neuronal replay in the hippocampus. This manipulation resulted in enhanced recall of card location the following day (Rasch et al., 2007). Spatial memory enhancement has also been observed when a trial-unique auditory cue paired with an object was re-presented during NREM sleep in humans (Rudoy et al., 2009). The presentation of the auditory cue during SWS increased activation of the medial temporal lobe and altered parahippocampal-medial prefrontal connectivity which has traditionally been associated with declarative memory (van Dongen et al., 2012). Not only has inducing neuronal replay during SWS been shown to enhance memory consolidation, but SPW-R disruption impairs hippocampus-dependent memory (Girardeau et al., 2009; Nokia et al., 2012). In 2012, Nokia and colleagues demonstrated the necessity of SPW-R hippocampal events that are characteristic of SWS by disrupting SPW-R with electrical stimulation in rabbits. This manipulation impaired trace eyeblink conditioning, a hippocampus-dependent learning task (Nokia et al., 2012). Other studies in humans have shown that reactivation during slow wave sleep can enhance not only spatial memory but procedural memories as well, suggesting that reintroduction of cues during sleep may reactivate other brain regions as well (Antony et al., 2012; Oudiette et al., 2013). These findings validate the hypothesis that NREM SWS is important for learning and memory (Girardeau et al., 2009; Ego-Stengel and Wilson, 2010).

Reports from human studies suggest that there is a dissociation between different sleep stages and the consolidation of different types of memory. In the 'dual hypothesis' of sleep, NREM sleep is responsible for improvements in declarative memory consolidation, while REM sleep plays a more significant role for procedural and emotional memory consolidation (Gais and Born, 2004). Declarative memory relies on the hippocampus, while procedural memory relies on striatal and cerebellar function (Squire et al., 1993; Doyon et al., 2003). Early studies demonstrated that NREM sleep improved declarative memories in humans. In the first set of studies to examine the role of NREM sleep in a declarative task, humans learned a verbal paired associates task before a sleep period known for high percentage of NREM sleep and low percentage of REM sleep. These subjects displayed superior memory in comparison to subjects trained before a high percentage of REM sleep. (Yaroush et al., 1971; Barrett and Ekstrand, 1972; Fowler et al., 1973). A later study by Plihal and Born found that subjects trained on a declarative task before sleep (predominantly composed of NREM sleep) and awakened 3 hours later exhibited higher rates of retention compared to individuals trained on the same task who slept during a period known for high REM sleep composition (Plihal and Born, 1997, 1999).

While the work mentioned above demonstrates the importance of NREM sleep to declarative memory, researchers have also observed that procedural memory (a type of non-declarative memory) benefits from REM sleep. Plihal and Born also examined the effects of REM sleep for procedural memory consolidation. Subjects who slept during a period dominated by REM sleep displayed more procedural memory gains than those who slept during a predominantly NREM sleep period (Plihal and Born, 1997, 1999). Although these studies established the importance of NREM sleep for declarative memory and REM sleep for procedural memory, the levels of cortisol, a stress hormone

that has the ability to interfere with learning and recall (Ackermann et al., 2013; Goerke et al., 2013), differed between those who were trained right before a high period of NREM sleep and those who were trained right before a high period of REM sleep (Plihal and Born, 1997, 1999). However, other studies have confirmed their findings by demonstrating this phenomenon of REM sleep-related memory enhancements after learning on various procedural tasks including priming and visuo-motor tasks (Mandai et al., 1989; Buchegger et al., 1991; Smith and Lapp, 1991; Smith and Smith, 2003; Wagner et al., 2003).

This 'dual hypothesis' paints a simplistic picture of how a particular stage of sleep potentially benefits one type of memory, while another sleep stage mediates the consolidation of other forms of memory. However, the sleep period known for a high percentage of REM sleep also contains a high percentage of stage 2 sleep and thus spindles. Therefore, procedural tasks could be benefiting from high spindle density, which have been associated with improvements in procedural memory, and not solely REM sleep (Tamaki et al., 2008, 2009; Rasch et al., 2009). Furthermore, other findings contradict this REM sleep-procedural memory link and NREM sleep-declarative memory link assertion. For instance, REM sleep has been shown to be important for emotional declarative memories (Wagner et al., 2001; Wagner, 2002). NREM sleep has also been found to play a role in procedural tasks further weakening the "dual hypothesis" argument (Walker et al., 2002, 2003). However, many of the procedural tasks developed for these experiments seem to require hippocampal involvement as well (Poldrack et al., 2001; Schendan et al., 2003). This hippocampal component of the task may require stages of NREM sleep to enhance procedural memory consolidation. This suggests that the design of the task matters to a great extent in order to control for activation of certain brain structures.

Clearly, the “dual hypothesis” dissociation between REM and NREM sleep in memory consolidation is more complex than the previously described straightforward examples. Other groups have posed alternative hypotheses that may account for the sleep stage-memory complexities, one such alternative is the “sequential hypothesis” (Giuditta et al., 1995). In this hypothesis, neither REM nor NREM sleep alone can account for memory consolidation, but the order of NREM and REM sleep after training as well as the transitions between NREM and REM sleep are essential for memory (Giuditta et al., 1995; Ambrosini and Giuditta, 2001).

Section 2. Sleep deprivation disrupts hippocampal function

2.1. Sleep deprivation disrupts memory consolidation

Many studies have utilized sleep deprivation to examine the role of sleep in memory consolidation. Researchers have developed several techniques for sleep deprivation in rodents to assess how sleep loss impairs memory. Some of the main methods of sleep deprivation include the “rotating platform” technique, the “flower-pot” technique, gentle handling, novel object introduction, and optogenetic stimulation. Each method has associated positive and negatives and has been discussed more thoroughly previously (see Havekes et al. 2012). Sleep deprivation administered after learning disrupts the consolidation period and impairs memories (Fishbein, 1971; Leconte et al., 1974; Linden et al., 1975). The hippocampus, in particular, appears to be vulnerable to this manipulation as demonstrated in hippocampal-dependent memory tasks after sleep deprivation. The first set of experiments to demonstrate this sensitivity used the Morris water maze task, which can be configured to either a hippocampus-dependent version or a hippocampus-independent version (Morris et al., 1982). Previously sleep deprived animals exhibited memory impairments in the hippocampus dependent version of the

task. However, sleep deprived animals subjected to the hippocampus-independent version did not demonstrate a memory impairment (Smith and Rose, 1996, 1997). This interesting dissociation between hippocampus-dependent and hippocampus-independent memory tasks was not restricted to the Morris water maze, but has been demonstrated with fear-conditioning tasks as well (LeDoux, 2000). Mice sleep deprived post-training exhibit memory impairments in the hippocampus-dependent configuration of this task, but not the hippocampus-independent configuration of the task (Graves et al., 2003a). Other studies examined sleep deprivation prior to learning in fear conditioning tasks and observed similar results (Bueno et al., 1994; McDermott et al., 2003; Ruskin et al., 2004; Ruskin and LaHoste, 2009). The Y-maze or T-maze is another type of dissociation task where researchers can examine the learning strategy animals employ to perform the task (Oliveira et al., 1997). This task allows researchers to assess whether sleep deprived animals shift from employing a spatial strategy (hippocampus-dependent) to a response strategy (hippocampus-independent) (Hagewoud et al., 2010b). Daily 5-hour sleep deprivation after training induced a shift from using a spatial strategy to a response strategy to navigate the maze (Hagewoud et al., 2010b). Watts and colleagues also demonstrated that decreased REM sleep as well as decreased spindle-rich transition to REM sleep by a norepinephrine reuptake inhibitor, desipramine (DMI), impaired performance in a hippocampus-dependent spatial task, while REM sleep suppression actually enhanced striatal-dependent configuration of the T-maze. This enhancement in striatal learning was likely due to the increased SWS that accompanied the pharmacological inhibition of REM sleep (Watts et al., 2012). These results confirm that the hippocampus is susceptible to the negative effects of insufficient sleep, especially reductions in sleep spindle density.

Studies have examined the effect of sleep deprivation during specific time windows of consolidation. Memory appears most sensitive to sleep deprivation when sleep is delayed after acquisition. However, if sleep occurs immediately after acquisition then long-term memory remains intact even if sleep is prevented at later time points, suggesting a sensitive period for sleep early during consolidation (Smith and Rose, 1996; Graves et al., 2003a; Gais et al., 2006; Palchykova et al., 2006). Based on rodent studies, this immediate window coincides with sensitive periods of molecular signaling, protein synthesis, and mRNA synthesis required for memory consolidation (Bernabeu et al., 1997a; Bourtchouladze et al., 1998; Igaz et al., 2002; Trifilieff et al., 2006). For instance, delaying sleep for 5 hours after acquisition impaired long-term memory in the contextual fear condition paradigm. However, immediate sleep followed by a later 5-hour period of sleep deprivation had no effect on long-term hippocampus-dependent memory (Graves et al., 2003a). This finding has also been observed within the hippocampus-dependent version of the water maze task (Smith and Rose, 1997). Subgroups of animals were sleep deprived during different times after training. Delaying sleep for the first 4 hours after training impaired memory in this task, whereas immediate sleep after learning did not impair memory (Smith and Rose, 1997). The effect of immediate versus delayed sleep after acquisition was also examined using the novel object recognition task (Palchykova et al., 2006). The beneficial effect of immediate sleep on memory has also been documented in humans performing a hippocampus-dependent declarative memory task (Gais et al., 2006). In a study by Gais and colleagues, one group was allowed immediate sleep after task acquisition at night, while sleep was delayed in the other group. Delaying sleep after task acquisition impaired performance in this task (Gais et al., 2006). These findings suggest the existence of a critical period for sleep deprivation to disrupt memory formation. This period occurs immediately after

acquisition, and overlaps with molecular signaling-sensitive time windows during the consolidation period. In **Chapter 2**, I explore the critical time window for sleep deprivation to disrupt consolidation-related processes.

2.2. Sleep deprivation impairs hippocampal synaptic plasticity

Sleep deprivation is detrimental to hippocampus-dependent memory. As the neural correlate of learning and memory, it is not surprising that sleep deprivation disrupts synaptic plasticity in the hippocampus as well. Long-term potentiation (LTP), a form of synaptic plasticity, is a long-lasting change in the strength of synaptic connections through the involvement of various molecular signaling cascades and, in some cases, protein synthesis (Bliss and Lomo, 1973; Whitlock et al., 2006). Campbell and colleagues examined LTP in area CA1 *in vitro* after 12 hours of total sleep deprivation, and found that the procedure inhibited induction of LTP in the hippocampus of rodents (Campbell et al., 2002). Since this study, follow-up studies have given us an in depth perspective on the effects of sleep deprivation on LTP. Researchers demonstrated that, similar to behavioral studies, LTP is vulnerable to total sleep deprivation, as well as REM-specific sleep deprivation and fragmented sleep (McDermott et al., 2003; Tartar et al., 2006; Ravassard et al., 2009; Florian et al., 2011). Similar LTP deficits occurred after sleep deprivation *in vivo* in dentate gyrus-CA3 region (Romcy-Pereira and Pavlides, 2004; Marks and Wayner, 2005; Ishikawa et al., 2006; Alhaider et al., 2011). The *in vivo* studies demonstrated that this LTP deficit was not an artifact of slice preparation but was a result of the influence of sleep deprivation on the intact hippocampal circuitry. The ability to induce LTP *in vivo* allowed researchers to investigate the effect of sleep deprivation on the maintenance phase of LTP., which exhibited impairment after total sleep deprivation and REM sleep deprivation in this

paradigm (Romcy-Pereira and Pavlides, 2004; Ishikawa et al., 2006). This suggests that sleep deprivation perturbs molecular signaling pathways underlying both the induction phase and the maintenance phase of LTP.

A limited number of studies have examined disrupted signaling pathways that underlie the deficit in LTP. Of those, even fewer have tried to rescue the phenotype. Work from our research group demonstrated that acute sleep deprivation by gentle handling specifically disrupted LTP requiring cAMP signaling. Our group went on to show that the LTP deficit induced by sleep deprivation could be rescued by increasing cAMP signaling (Vecsey et al., 2009). More recent work showed that LTP was resistant to sleep deprivation if extracellular adenosine was attenuated either using a pharmacological approach or genetic approach (Alhaider et al., 2010a; Florian et al., 2011). These studies established cAMP and adenosine as playing a role in the LTP deficit caused by sleep deprivation. There are additional cellular signaling mechanisms that are also altered by sleep loss. Other studies have examined the contribution of N-methyl-D-aspartate (NMDA) receptor function in the impairment of LTP after sleep deprivation. An extended period of sleep deprivation for 24-72 hours affected NMDA receptor composition and attenuated receptor function, leading to a disruption in both induction and maintenance of LTP. This LTP deficit was reversed by treatment with an NMDA receptor NR1 subunit agonist, glycine (McDermott et al., 2006). This finding suggests disturbances in NMDA receptor function can lead to the LTP deficits observed after chronic periods of sleep deprivation.

The effects of sleep deprivation have also been examined in long term depression (LTD), which is another form of hippocampal synaptic plasticity requiring signaling components different from LTP. In contrast to the attenuation of LTP by sleep deprivation, researchers have either observed no change or facilitation of LTD after

sleep deprivation in the hippocampus (McDermott et al., 2003; Tadavarty et al., 2009, 2011; Yang et al., 2012). This discrepancy in the effect of sleep deprivation on LTD facilitation is likely due to experimental design differences in LTD induction protocol as well as different sleep deprivation manipulations. For instance, while Tadavarty and colleagues used gentle handling to sleep deprive animals allowing them to examine the circadian contribution, McDermott and colleagues used the multiple platform method to have a 72-hour period of REM sleep deprivation (McDermott et al., 2003; Tadavarty et al., 2009). Similarly, Yang and colleagues examined circadian contribution along with sleep pressure on the resulting LTD (Yang et al., 2012). Additionally, *in vitro* preparations were used in earlier cases of observed LTD facilitation after sleep deprivation (Tadavarty et al., 2009, 2011). However, facilitation of LTD *in vivo* after sleep deprivation has recently been observed with elevated sleep pressure due to a combination of sleep deprivation and time of day, further validating the effect of sleep deprivation on LTD facilitation (Yang et al., 2012). Tadavarty and colleagues examined the signaling pathways underlying facilitation of LTD in response to sleep deprivation. They found increased reliance on the $GABA_B$ receptor and metabotropic Glutamate 1α receptors, while NMDA receptors did not play a role (Tadavarty et al., 2011). This suggests that sleep deprivation has different effects on the signaling pathways underlying these two opposing forms of plasticity.

LTP induced *in vitro* and *in vivo* displays a graded sensitivity to sleep deprivation. Extended periods of sleep deprivation for 24-72 hours appear to eliminate or reduce LTP induction *in vitro* (Campbell et al., 2002; McDermott et al., 2003). However, brief periods of sleep deprivation only appear to disrupt signaling underlying LTP maintenance while induction remains intact (Vecsey et al., 2009; Florian et al., 2011). No studies have specifically examined the time course of sleep deprivation to impair LTP. However, Kopp

and colleagues have demonstrated that as little as 4 hours of sleep deprivation can impair LTP *in vitro* (Kopp et al., 2006). In terms of *in vivo* experiments that have examined the time course of sleep deprivation, Marks and Wayner found that 3, 6, and 9 hours of sleep deprivation impaired LTP, demonstrating that even an acute 3 hour-period of sleep deprivation is sufficient to impair synaptic plasticity (Marks and Wayner, 2005). Critically, however, the effect of sleep deprivation on hippocampal LTP during a period of active memory consolidation has not previously been examined. In **Chapter 2**, I explore the time window during consolidation for sleep deprivation to disrupt LTP. By assessing hippocampal LTP after training, we can determine the effects of sleep loss on hippocampal plasticity during the period of consolidation. Prior to these experiments, the contribution of sleep deprivation to LTP during the consolidation stage of memory had yet to be examined. In conclusion, sleep deprivation perturbs hippocampal plasticity even after brief bouts of sleep deprivation. These studies suggest that specific disruptions in molecular signaling by sleep deprivation impair LTP, as well as related behavioral phenotypes observed. Some of the known sleep deprivation-induced changes in signaling will be discussed in the following section.

2.3. Sleep deprivation disrupts hippocampal signaling necessary for memory

Sleep deprivation disrupts multiple signaling pathways in the hippocampus in parallel that lead to plasticity and memory impairments. This section outlines some of the more well-known signaling targets sensitive to sleep loss.

N-methyl-*D*-aspartate (*NMDA) Receptor and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) Receptor*

NMDA receptor activity plays a significant role in all 3 stages of memory, most notably, the consolidation phase where the memory advances from a labile form to a

more stable permanent form (Hernandez and Abel, 2011). These receptors allow the expression of LTP through increased influx of Ca^{2+} (Xia and Storm, 2012). Sleep deprivation has been shown to impair proper activation of this glutamate receptor-type through altering receptor subunit composition, surface expression, and reduced Ca^{2+} influx (Chang et al., 2012). McDermott and colleagues found that prolonged sleep deprivation (72 h) reduced the NMDA/AMPA ratio in CA1 pyramidal cells in response to Schaffer collateral stimulation. NMDAR-mediated currents from the distal dendrites of CA1 cells had reduced amplitude after sleep deprivation manipulation. This was most likely due to the reduced surface expression of NMDA receptors after sleep deprivation (McDermott et al., 2006). The same research group also observed a higher proportion of NR1 and NR2A subunits of the NMDA receptor located intracellularly compared to surface level after sleep deprivation (McDermott et al., 2006). This disruption in NMDA receptor trafficking to the cell surface and reduction in NMDAR-mediated current was also observed with 24 hours of sleep deprivation (Chen et al., 2006). Other groups have also observed decreases in NR1 protein expression in the hippocampus after sleep deprivation, supporting the hypothesis that the NMDA receptor is an important molecular target for sleep deprivation (Ravassard et al., 2009; Chang et al., 2012). NR1 subunit decrease was accompanied by synaptic plasticity and memory deficits that could be rescued with pharmacological treatment of glycine, an NR1 agonist (McDermott et al., 2003; Chen et al., 2006). The NR2B subunit of the NMDA receptor has also been observed to decrease in the hippocampus as a result of REM sleep deprivation (Lopez et al., 2008; Park et al., 2012). Although both of these studies observed clear differences in trafficking and NMDAR-mediated current using extended periods of sleep deprivation, these findings conflict with findings from briefer periods of sleep deprivation (Vecsey et al., 2009). Kopp and colleagues found that 4 hours of sleep deprivation by exposure to a

novel environment and introduction to new nesting material increased the NR2A/NR2B NMDA-receptor subunit ratio as well as total NR2A subunits in the hippocampus using electron microscopy (Kopp et al., 2006; Longordo et al., 2009). This finding was correlated with a shift in the frequency dependence needed to elicit LTD and LTP, decreasing the threshold frequency to induce LTD and increasing the threshold frequency to induce LTP. This group also observed that removal of NR2A subunits prevented the synaptic plasticity changes induced by sleep deprivation (Longordo et al., 2009). However, these differences in subunit ratios and mediated current after sleep deprivation have not been observed by other research groups. After 5 hours of sleep deprivation by the gentle handling procedure (Ledoux et al., 1996), no difference was observed in NMDA receptor-mediated current or in the NMDAR/AMPAR ratio in CA1 (Vecsey et al., 2009). These differences in NMDA receptor-mediated current could be attributed to sleep deprivation techniques used in these two experimental designs. In conclusion, NMDA receptor function is needed for plasticity and memory. Longer periods of sleep deprivation disrupt NMDA receptor function, impairing both plasticity and memory. Meanwhile, more acute sleep deprivation may or may not have this effect on receptor function depending on the sleep deprivation technique.

Sleep-wake homeostasis also alters the expression of AMPA receptors, another class of glutamate receptors involved in memory, in cortical regions (Cirelli and Tononi, 2000). Cortical and hippocampal AMPA receptor levels increase during waking hours and decrease over the sleeping period (Vyazovskiy et al., 2008). Although AMPA receptors appear to be under the influence of sleep homeostatic processes, sleep deprivation was previously not thought to interfere with hippocampal AMPA receptor function (McDermott et al., 2006). However, more recent studies showed that sleep deprivation modulated the function and efficacy of AMPA receptors in both the cortex

and hippocampus. Ravassard and colleagues observed that multiple days of REM sleep deprivation reduced AMPA receptor function in the hippocampus. Specifically, they found decreased protein expression of the AMPA receptor GluA1 subunit as a result of sleep deprivation (Ravassard et al., 2009). Other work has shown similar results, finding a reduction in GluA1 in the hippocampus after REM sleep deprivation (Lopez et al., 2008). This subunit in particular has been linked to spatial memory (Schmitt et al., 2005), suggesting that reduced GluA1 expression due to sleep deprivation could possibly explain the impaired spatial memory. To further assess this subunit in sleep deprivation, Hagewoud and colleagues examined the effect of a 12-hour sleep deprivation period on total hippocampal GluA1 protein expression and phosphorylation of GluA1-serine 845 site, an important step for incorporation of the receptor into the membrane. In this study, 12 hours of sleep deprivation did not decrease total protein levels of hippocampal GluA1, however this sleep deprivation manipulation decreased phosphorylation of the serine 845 site (Hagewoud et al., 2010a). Although these studies demonstrated reduced AMPA receptor function, other contradictory findings from Vyazovskiy and colleagues observed increased levels of total GluA1 in the hippocampus and cortex after enforced wakefulness (Vyazovskiy et al., 2008). This difference could be a result of animal strain. In their study, they chose to use the Wistar Kyoto rat strain that is a well-known genetic animal model of depression. This may have confounded their study because short-term sleep loss has been shown to improve symptoms of depression and increase hippocampal neurogenesis (Grassi Zucconi et al., 2006). Overall, there is a disruption in AMPA receptor function after sleep deprivation due to either altered protein expression of GluA1 and in some cases reduced phosphorylation of a site on GluA1 necessary for AMPA receptor membrane insertion. Disruption of AMPA receptor function contributes to spatial memory deficits observed in tasks after sleep deprivation.

Glutamate

As described in the previous section, both glutamate NMDA receptors and AMPA receptors fluctuate through the sleep-wake cycle. Not surprisingly, the ligand for these receptors, glutamate, has also been implicated in sleep-wake homeostasis (Disbrow and Ruth, 1984; Mukherjee et al., 2012). Limited studies have examined glutamate levels in the hippocampus following sleep deprivation. One study that examined the effects of sleep deprivation on glutamate in the hippocampus found that glutamate levels increase after sleep deprivation (Cortese et al., 2010). Of the studies that have examined effects of sleep on glutamate, most concentrate on the effects of sleep and sleep loss in the cortex. These studies have observed that glutamate levels fluctuate progressively through sleep/wake states in the cortex (Jasper et al., 1965; Lopez-Rodriguez et al., 2007). Few have examined the effects of sleep deprivation on cortical glutamate levels. Bettendorff and colleagues found that after 12-24 hours of REM sleep deprivation glutamate levels increased (Bettendorff et al., 1996). Contradicting these findings, Wang and colleagues found that 96 hours of REM sleep deprivation did not affect glutamate levels in the cortex (Wang and Li, 2002). While these studies used microdialysis to obtain glutamate measurements, another study using *in vivo* amperometry observed increased glutamate levels in cortical areas during extended wakefulness and during REM sleep and decreased glutamate levels during SWS (Dash et al., 2009). These findings become more complex as previous sleep-wake history of the animals also factors into these results. The authors also examined the effects of sleep deprivation on cortical glutamate levels. Initially, if the animal is sleep deprived, glutamate levels will increase in the first hour of sleep deprivation. However, as the sleep deprivation continues, glutamate levels will begin to decline after 3 hours. During this period of declining glutamate, attempts to sleep are the highest (Dash et al., 2009).

Adenosine and Astrocytes

Different research groups have hypothesized that increased sleep pressure correlates with elevated adenosine tone, resulting in increased intensity of future sleep episodes (Bjorness and Greene, 2009; Porkka-Heiskanen and Kalinchuk, 2011). Adenosine is a key neuromodulator highly implicated in the sleep-wake literature (Basheer et al., 2004). Adenosine levels are known to fluctuate throughout the day, peaking during the height of the active period and then diminishing over the animal's resting period in both the hippocampus and the neostriatum (Huston et al., 1996). This fluctuation in adenosine over the sleep-wake cycle has also been observed in the forebrain of animals (Porkka-Heiskanen, 1997). Studies have observed both this natural homeostatic oscillation of adenosine as well as an increase in adenosine signaling with extended periods of wakefulness (Porkka-Heiskanen, 1997; Basheer et al., 2007; Elmenhorst et al., 2007). The increase in adenosine after periods of wakefulness may contribute to the increased drive for sleep. The effect of heightened sleepiness as a result of elevated adenosine levels can be reproduced by pharmacologically increasing adenosine (Porkka-Heiskanen, 1997). Increased adenosine due to sleep deprivation contributes to activation of the adenosine A1 receptor, which inhibits synaptic transmission through attenuation of activity from neighboring excitatory neurons (Brundege and Dunwiddie, 1996; Haas and Selbach, 2000; Hargus et al., 2009). Activation of the A1 receptor also inhibits cAMP signaling through G_i protein coupling, intersecting with another disrupted pathway (Haas and Selbach, 2000; Fredholm et al., 2005). The actions of adenosine on the A1 receptor within the hippocampus could account for the negative contribution of sleep deprivation to memory. To examine this possibility, adenosine A1 receptors were pharmacologically inhibited with 8-cyclopentyl-1,3,dimethylxanthine (CPT) in animals sleep deprived post-training (Halassa et al., 2009;

Florian et al., 2011). CPT infusion into the hippocampus rescued memory and plasticity impairments induced by sleep deprivation (Florian et al., 2011). Blocking release of transmitters from astrocytes, a source of adenosine, also rescued memory and plasticity impairments (Halassa et al., 2009). These studies support the involvement of adenosine and A1 receptor signaling in the negative consequences induced by sleep deprivation.

cAMP-PKA-PDE4

Hippocampal activation of cAMP and PKA signaling are known to be important for memory consolidation as previously discussed in the "Molecular signaling consolidates memory" section of this **Chapter**. Both of these molecular targets have also previously been implicated in sleep processes (Graves et al., 2001; Hendricks et al., 2001; Hellman et al., 2010; Luo et al., 2013). Sleep deprivation interferes with this hippocampal signaling pathway in electrophysiology studies (Vecsey et al., 2009). The observation that shorter periods of sleep deprivation disrupt LTP maintenance suggested that sleep deprivation perturbs signaling pathways specific to the maintenance phase of LTP (Romcy-Pereira and Pavlides, 2004; Vecsey et al., 2009; Florian et al., 2011). Vecsey and colleagues examined molecular signaling required for late-phase LTP (L-LTP) maintenance and observed that brief sleep deprivation reduced both cAMP and PKA activity (Vecsey et al., 2009).

As a result of cAMP and PKA disruption by sleep deprivation, hippocampal downstream targets in this pathway are also perturbed. For instance, the aforementioned phosphorylation of serine 845 in the AMPA receptor subunit GluA1 by PKA has been shown to be altered in 3 studies examining the contribution of sleep deprivation to molecular signaling (Vyazovskiy et al., 2008; Ravassard et al., 2009; Hagewoud et al., 2010a). Interestingly, while moderate to chronic sleep deprivation

reduced phosphorylation of serine 845 (Ravassard et al., 2009; Hagewoud et al., 2010a), Vyazovskiy and colleagues found an increase in phosphorylation of serine 845 after 4 hours of enforced wakefulness (Vyazovskiy et al., 2008). This difference could be due to both the length of sleep deprivation as well as the sleep deprivation technique used in these experiments. Another downstream target in the cAMP-PKA pathway is the transcription factor CREB, which has already been described as important for memory and plasticity and is also affected by sleep deprivation. Phosphorylation of CREB by PKA at Serine 133 is reduced in the hippocampus as a result of sleep deprivation (Vecsey et al., 2009; Zhao et al., 2010; Alhaider et al., 2011). Sleep deprivation decreased phosphorylation of CREB in the amygdala, a brain region that receives contextual inputs from the hippocampus, and is important for emotionally-laced memories (Pinho et al., 2013). In this experiment, in contrast to the experimental design used in the study by Vecsey and colleagues, animals were sleep deprived for 72 hours by multiple platform method and then trained in the fear conditioning task. This extensive sleep deprivation procedure along with fear conditioning may elevate stress, which is known to affect phosphorylation of CREB, to a greater extent than gentle handling alone (Xu et al., 2006).

Phosphodiesterase 4 (PDE4) is the enzyme responsible for degradation of hippocampal cAMP, thereby reducing PKA activity. Vecsey and colleagues also found increased activity of PDE4 and protein expression of the specific PDE4 isoform, PDE4A5, after sleep deprivation. They found that blocking PDE4 signaling during sleep deprivation rescued not only LTP deficits due to sleep deprivation, but also the sleep deprivation induced deficits in hippocampus-dependent memory consolidation (Vecsey et al., 2009). These experiments provided further evidence that the cAMP-PKA pathway and its downstream components are key targets for disruption by sleep deprivation.

Moreover, rescue by PDE4 inhibition demonstrates that this molecular disruption by sleep deprivation produces the functional deficits in behavior and plasticity. I further investigate PDE4A as a mediator for the effects of sleep deprivation on hippocampal function in **Chapter 3**. In addition, it appears that PDE4A5 may regulate cAMP through interaction with the neurotrophin receptor $p75^{NTR}$ (Sachs and Akassoglou, 2007; Sachs et al., 2007). I examine the effect of this partnering receptor on sleep-deprivation-induced impairments in the **Appendix**.

Conclusion

Within our society, working around the clock is applauded and seen as a form of dedication. It is commonly viewed as a driving force for success. Due to this mindset, sufficient sleep often falls by the wayside. One recurring theme that has surfaced from sleep research is that even minimal sleep loss contributes to less than stellar cognitive performance. Time windows exist where sleep deprivation disrupts hippocampal function. As mentioned previously, the time period most critical for memory formation appears to occur somewhere within the first six hours following learning. Sleep deprivation constrained to this period after learning is sufficient to disrupt consolidation dependent on the hippocampus (Smith and Rose, 1997; Graves et al., 2003a; Palchykova et al., 2006). However, the exact onset and end of these windows where sleep is necessary for hippocampus-dependent memory have not been clearly defined. This is the focus of **Chapter 2**, as identification of the temporal parameters of sleep necessary for memory consolidation will enable a better understanding of the effects of both sleep and sleep loss on memory.

So how do we currently counter the effects of sleep deprivation, which affects so much of the population? In terms of treatment options, development of new therapeutics

is severely lacking in the ability to battle the negative cognitive effects that accompany insufficient sleep. Many of the drugs that exist have uncharacterized mechanisms of action. Caffeine, one of the most common stimulants, is still considered the top over the counter drug to combat tiredness. Caffeine enhances alertness by antagonism of adenosine A1 receptor and increases cAMP signaling through inhibition of phosphodiesterase (Fredholm et al., 1999; Wu et al., 2009). Chronic caffeine administration has been shown to prevent sleep loss-induced impairment of cognitive function and synaptic plasticity (Alhaider et al., 2010b). Another stimulant, modafinil, prescribed for treatment of excessive daytime sleepiness prevents sleep deprivation induced cognitive impairments (Moreira et al., 2010). This drug has not been as well characterized, but is thought to involve orexinergic neurons that project to many areas that regulate wakefulness (Chemelli et al., 1999a; Scammell et al., 2000). Although the orexinergic system is involved in the stimulant effects of modafinil, this system is not the only pathway that modafinil works through to increase alertness and rescue cognition (Gerrard and Malcolm, 2007). Other neurotransmitter systems affected by modafinil include histamine, norepinephrine, serotonin, dopamine and gamma-aminobutryic acid (GABA). The GABA neurotransmitter system has come to the forefront as a possible target for therapeutic treatment. Zolpidem (Ambien), a $GABA_A$ receptor agonist, is a drug recently assessed for its effect on sleep features and resulting hippocampus-dependent memory consolidation. This drug seemingly enhances memory consolidation by increasing sleep spindle density, and decreasing REM sleep, (Mednick et al., 2013). Although these drugs aid in treating the cognitive impairments induced by sleep deprivation, none of the current treatment options effectively treat the underlying physiology of sleep deprivation, but instead seem to mask the issue by treating peripheral symptoms accompanying sleep loss. Understanding the mechanics of

molecular pathways involved in sleep deprivation will increase the likelihood of creating more effective treatment options in the future. In this vein, I examine some potential molecular targets of sleep deprivation in **Chapter 3** and the **Appendix**.

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Author Contributions

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FIGURE 1.

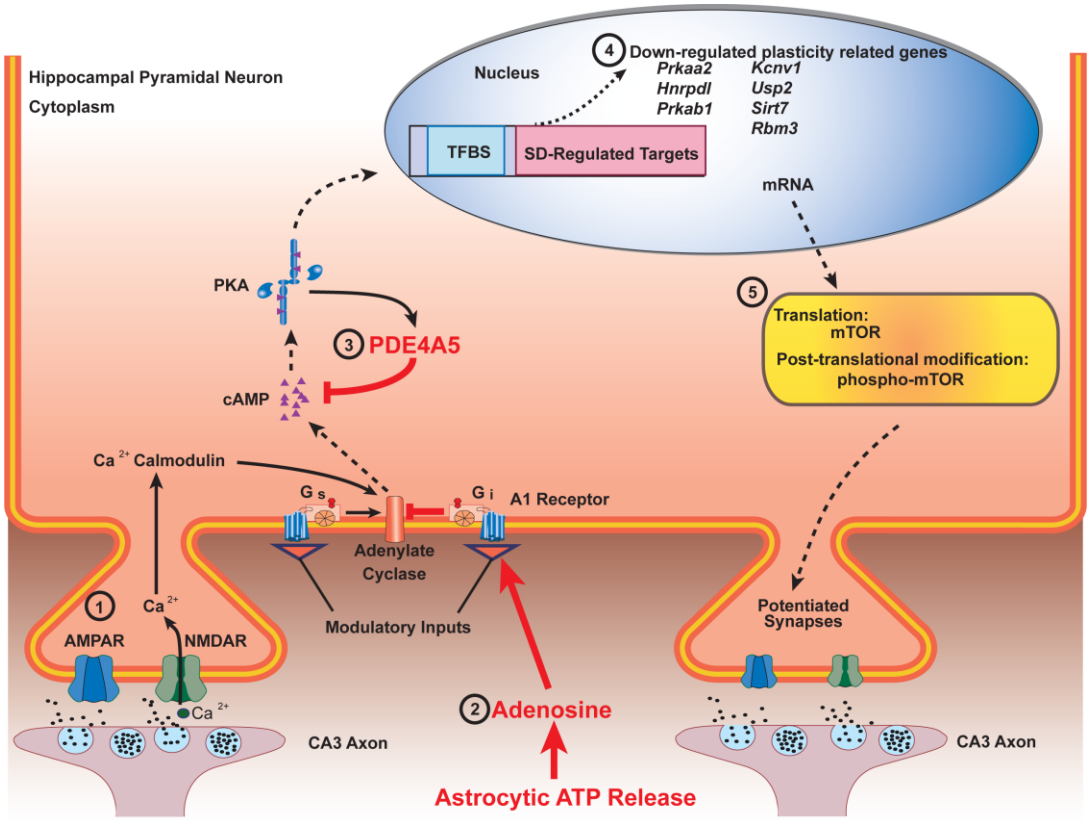

Figure 1. *A schematic overview of hippocampal signaling pathways following sleep deprivation.*

Studies suggest multiple mechanisms by which sleep deprivation disrupts memory consolidation. 1. Sleep deprivation modulates glutamatergic signaling through alterations in NMDA and AMPA receptor subunit composition. This disruption in receptor function attenuates molecular signaling cascades through reduced calcium influx. 2. Sleep deprivation raises extracellular adenosine, an endogenous sleep factor, and astrocytes are one source of adenosine. Adenosine acts through the adeonsine A1 receptor to reduce plasticity by enhancing the inhibitory tone in the hippocampus. 3. Elevation of phosphodiesterase 4 A by sleep deprivation perturbs cAMP intracellular signaling. 4. Sleep deprivation decreases transcription of plasticity related genes. 5. Sleep deprivation down-regulates mTOR signaling, a key regulator of protein synthesis required for memory consolidation. Red arrows indicate an increase of the signaling pathway. Red font indicates an increase of activity. Dashed black arrows indicate an attenuation of the signaling pathway. Adapted with permission, from (Hernandez and Abel, 2011).

CHAPTER TWO

SLEEP DEPRIVATION DURING A SPECIFIC 3-HOUR TIME WINDOW POST TRAINING IMPAIRS HIPPOCAMPAL SYNAPTIC PLASTICITY AND MEMORY

Abstract

Sleep deprivation disrupts hippocampal function and plasticity. In particular, long-term memory consolidation is impaired by sleep deprivation, suggesting that a specific critical period exists following learning where sleep is necessary. However, the temporal parameters of sleep deprivation that disrupt hippocampal function have not been explored. To elucidate the impact of sleep deprivation on long-term memory consolidation and synaptic plasticity, long-term memory was assessed when mice were sleep deprived following training in the hippocampus-dependent object place recognition task. We found that 3 hours of sleep deprivation significantly impaired memory when deprivation began 1 hour after training (ZT 2-5). In contrast, 3 hours of deprivation beginning immediately post-training did not impair spatial memory. Furthermore, delayed 3-hour sleep deprivation after training also impaired hippocampal long-term potentiation (LTP), whereas sleep deprivation immediately after training did not affect LTP. Together, our findings define a specific 3-hour critical period, from ZT 2-5, during which sleep deprivation impairs hippocampal function.

Introduction

Despite the fact that sleep exposes an organism to increased risk of attack or predation due to time spent unaware of its surroundings, sleep is an evolutionarily conserved phenomenon that is critical for survival. Inadequate sleep contributes to both physical and mental exhaustion and deterioration. Modern society suffers from unprecedented rates of sleep loss. According to the Center for Disease Control, 7-19% of adults in the US report inadequate sleep, and an estimated 70 million Americans suffer from chronic sleep disorders. Although the vital function of sleep has yet to be determined, lack of sleep is detrimental to cognitive function.

One of the most notable negative consequences of sleep loss is impaired memory (Harrison and Horne, 2000). Memory is composed of at least three stages; acquisition, consolidation, and retrieval (Abel and Lattal, 2001). The effects of sleep deprivation have been examined on both the acquisition and consolidation of memory. Early studies explored the effects of sleep deprivation on memory acquisition (Stern, 1971), and it has since been repeatedly demonstrated that chronic sleep deprivation impairs acquisition (learning) (Youngblood et al., 1997; Chee and Choo, 2004; Durmer and Dinges, 2005; Van Der Werf et al., 2009; Hagewoud et al., 2010a; Havekes et al., 2012; Abel et al., 2013; Prince and Abel, 2013). More recently, however, multiple laboratories have explored the effects of acute sleep deprivation and sleep fragmentation during consolidation, showing that consolidation benefits from sleep and is hindered by sleep loss (Graves et al., 2003a; Vecsey et al., 2009; Hagewoud et al., 2010b, 2010c; Florian et al., 2011; Rolls et al., 2011; Inostroza et al., 2013).

Consolidation in hippocampus-dependent memory tasks is particularly sensitive to sleep loss. Sleep deprivation-induced deficits have been described for associative memory tasks such as contextual fear conditioning and for spatial memory tasks such as

the Morris water maze task and the object-place recognition (OPR) task, which is used in the present study (Smith and Rose, 1996, 1997; Graves et al., 2003a; Florian et al., 2011; Binder et al., 2012). OPR, in particular, is an ideal paradigm for examining the effects of sleep deprivation on hippocampal function because it is comparable to tasks that test declarative memory in humans, it is dependent on the hippocampus, and it is not aversive (Bussey et al., 2000; Mumby et al., 2002; Winters et al., 2004, 2008; Shrager et al., 2007; Oliveira et al., 2010).

Several studies have assessed whether sleep deprivation during specific time windows after training affects long-term memory (Smith and Rose, 1997; Graves et al., 2003a; Palchykova et al., 2006). In these studies, a sleep deprivation sensitive window, within the first 4-6 hours of consolidation, has been demonstrated in the consolidation of contextual memory, object recognition memory, and spatial memory. Memory is resistant to the effects of sleep deprivation if animals are sleep deprived after this window (Smith and Rose, 1997; Graves et al., 2003a; Palchykova et al., 2006). These findings suggest that there is a critical period during which memory is vulnerable to the effects of sleep deprivation. However, little work has been conducted to examine the precise timing of this sensitive time window. The aim of this study was to define the temporal parameters for the impact of sleep deprivation on memory consolidation.

Aside from the effects of sleep deprivation on behavioral measures of memory, sleep deprivation also disrupts synaptic plasticity, a neural correlate of memory. Campbell and colleagues demonstrated that 12 hours of sleep deprivation impairs hippocampal long-term plasticity (LTP), a form of synaptic plasticity (Campbell et al., 2002). LTP deficits have been observed *in vitro* after 4-5 hours of sleep deprivation as well (Kopp et al., 2006; Vecsey et al., 2009). The impact of short periods of sleep deprivation is specific to late-phase LTP (L-LTP), which requires protein synthesis and

the cyclic adenosine mono-phosphate (c-AMP) signaling pathway (Vecsey et al., 2009). Critically, however, the effects of sleep deprivation on hippocampal LTP during a period of active memory consolidation have not previously been examined. By assessing hippocampal LTP following training, in the sensitive window for sleep deprivation, we aimed to more accurately determine the effects of sleep and sleep loss on hippocampal plasticity associated with memory consolidation.

Previously, we demonstrated that as little as 6 hours of sleep deprivation immediately after task training disrupts long-term spatial memory in OPR (Florian et al., 2011). Here we aim to better define the critical period during which sleep is essential for hippocampal memory consolidation. By sleep depriving mice during different time windows, we demonstrate that as few as 3 hours of sleep deprivation during consolidation can affect both long-term memory and LTP.

Materials and Methods

Mice

One hundred and seventy-two C57BL/6J adult male mice (2 to 4 months of age) were pair housed and kept on a 12h/12h light/dark schedule with lights on at 7:00 AM (ZT 0). Food and water were available *ad libitum* throughout the experiments. All experiments were approved by the Institution of Animal Care and Use Committee of the University of Pennsylvania and were carried out in accordance with all National Institutes of Health guidelines.

Sleep Deprivation

To assess the effects of sleep deprivation (SD) on memory, mice (n = 92) were sleep-deprived using the gentle handling technique involving manual cage tapping, cage shaking, nestlet disturbance, and gentle animal prodding (Ledoux et al., 1996; Vecsey et

al., 2013). Prior work using electroencephalographic recordings has shown that this procedure effectively retains animals in a state of wakefulness for several hours (Meerlo et al., 2001). The frequency of these manipulations was monitored throughout the sleep deprivation period (**Fig. 2.8. A** and **2.8. B**). Separate groups of mice were sleep deprived for varying periods of SD after behavioral training as described in **Fig. 2.1. A** and **2.1. B**. Non-sleep deprivation (NSD) time-matched control groups were used for comparison with the SD experimental groups.

Object-place recognition (OPR)

For this task, we used a previously established design that has been shown to be hippocampus dependent (Havekes et al., 2012; Oliveira et al., 2010). Mice (n = 31) were handled for 2 minutes each day, for 6 consecutive days leading up to experimentation. The task was conducted in a grey rectangular box (40 cm x 30 cm x 30 cm) built of polyvinyl chloride plastic. Mice were placed in the empty box for 6 minutes for habituation. Mice were then removed and placed back in the home cage. After 3 minutes, mice were placed in the box with 3 different objects (a 100 ml glass bottle, a white cylinder, and a metallic rectangular tower) for 3 consecutive 6-minute training sessions. Each training session was separated by a 3-minute interval during which the animals were returned to the holding cages. At completion of the training sessions, NSD mice were left undisturbed in their home cages and SD mice were deprived of sleep by gentle handling. Twenty-four hours following the training session, mice were re-introduced to the spatial context in a single test session. In this session, one of the objects was repositioned (the displaced object: DO), thereby changing the spatial configuration of the objects in the box. Mice were allowed to explore objects for 6 minutes. Exploration was recorded during training and testing on a digital camera for

subsequent scoring of time spent exploring objects. Exploration of the objects was defined as the amount of time mice were oriented toward an object with their noses within 1 cm of it, and was measured by an experimenter who was blinded to the experimental history of the mouse.

The change in preference of object exploration between the last training session and test session was used as an index for spatial memory. Preference for an object during the training and test session was determined by calculating the percentage of time spent exploring a particular object versus total time spent exploring all objects. The formula for this measure is as follows: [TEST (DO/(DO + NDO) x 100]- [3rd TRAINING SESSION (DO/(DO+NDO) x 100], where DO = time spent exploring the displaced object and NDO = time spent exploring the non-displaced object. A positive change in percentage exploration of displaced object between test session and the final training session indicates preference for exploring displaced object. This is indicative of an accurate memory for the original locations of objects and that the mice were able to recognize the object's displacement. No change or a negative change in time spent exploring the displaced object in the test session compared to the last training session indicates inability to recognize the new spatial configuration.

Electrophysiology

To assess the effects of SD after OPR training on hippocampal LTP, electrophysiological recordings were performed as described previously (Vecsey et al., 2009). Animals were trained in the OPR paradigm at ZT 0 (n = 19). They were then either left undisturbed in their home cages (NSD control group, n = 9), or subjected to 3 hours of SD. Animals were sleep deprived for 3 hours either directly after training (OPR + SD ZT 1-4 group, n = 5) or 1 hour after training.(OPR + SD ZT 2-5 group n = 5) (**Fig.**

2.1. B). A separate group of naïve mice that did not receive OPR training were either sleep deprived from ZT 2-5 (SD ZT 2-5 group, n = 6) or left undisturbed in their home cages (NSD group, n = 8). Immediately after sleep deprivation, SD and time-matched NSD mice were sacrificed by cervical dislocation, and their hippocampi were dissected rapidly in iced oxygenated artificial cerebrospinal fluid (aCSF; 124 mM NaCl, 4.4 mM KCl, 1.3 mM $MgSO_4*7H_2O$, 1 $NaH_2PO_4*H_2O$, 26.2 mM $NaHCO_3$, 2.5 mM$CaCl_2*2H_2O$, 10 mM glucose). 400 μm thick transverse hippocampal slices were prepared using a Stoelting tissue chopper (Stoelting Co. Wood Dale, IL) and placed in an interface chamber and continuously perfused with oxygenated aCSF while they equilibrated for at least 1.5 hours at 28.0ºC before starting electrophysiological recordings. Single pathway recordings were made using a single bipolar stimulating electrode (A-M Systems; 0.002 inches diameter nichrome wire) placed in the stratum radiatum of the CA1 subfield to elicit action potentials in the axons of CA3 pyramidal neurons. An aCSF-filled glass microelectrode (A-M Systems; 1.5 mm x 0.85 mm) with a resistance between 0.5 and 3 MΩ placed in the stratum radiatum region of CA1 was used to record the resulting field excitatory post-synaptic potential (fEPSP). Data were acquired and analyzed using Clampex 8.2 software (Molecular Devices, Palo Alto, CA). Peak fEPSP amplitude was required to be at least 5 mV, and stimulus intensity was set to produce 40% of the maximal response. Test stimulation occurred once every minute. A 20-minute baseline period was recorded in each experiment prior to stimulation. To examine late-phase LTP (L-LTP), 4 trains of stimuli at 100 Hz for 1 second were delivered 5 minutes apart, otherwise known as spaced 4-train high frequency stimulation. Recordings continued for at least 2 hours after LTP induction. Initial fEPSP slopes were normalized against the average of the 20 baseline traces. Input-output characteristics in area CA1 were investigated by recording the fEPSPs elicited by stimuli of increasing intensity after

maximum fEPSP was determined. The initial fEPSP slopes were plotted against the amplitudes of corresponding presynaptic fiber volleys and fit with linear regressions. The maximum elicited fEPSP slope was also recorded as a measure of synaptic strength. Paired-pulse facilitation, a short-term form of synaptic plasticity and a measure of presynaptic function, was measured in slices from NSD and SD groups. Paired stimuli were delivered with varying interpulse intervals (300, 200, 100, 50, and 25 ms) and the fEPSP slope from the 2nd stimulus was plotted relative to the slope from the 1st stimulus to give the facilitation ratio. We combined NSD control data from the 2 experimental groups as they were not significantly different from each other.

2.5. Polysomnography

To determine if undisrupted sleep during these 2 time periods differed, sleep was measured using polysomnography. Naïve mice (n = 6) had electrodes implanted to record EEG and EMG signals and assess time spent in wakefulness, NREM, and REM sleep.

Adult male C57BL/6J mice were implanted with electroencephalographic (EEG) and electromyographic (EMG) electrodes under isoflurane anesthesia. EEG electrodes were implanted 1.5 mm bilateral to midline and 1.5 mm posterior to bregma on each side using an electric drill. A reference EEG electrode was placed 1.5 mm posterior to lambda over the cerebellum (Huber et al., 2000; Hellman et al., 2010; Vecsey et al., 2013). EMG electrodes were attached to nuchal musculature. Electrodes were held in place with glass ionomer resin (Ketaccem, 3M, Maplewood, MN, Chemelli et al., 1999). Electrodes consisted of Teflon coated wires (Cooner wires, Chatsworth, CA) soldered to gold socket contacts (Plastics One, Roanoke, VA) and pushed into a 6-pin plastic plug (363 plug, Plastics One), which was then attached to a commutator (SLC6, Plastics One;

(Veasey et al., 2000)). All recordings were analyzed with the right EEG electrode and were referenced to the cerebellum electrode. This electrode-referencing scheme is ideal for detecting alterations in delta and theta activity in mice (Franken et al., 2000; Tafti et al., 2003). Mice were housed individually and allowed 2 weeks of recovery after surgery. During the second week of recovery, mice were acclimated to the recording chambers and to the cables. During the second week, mice were handled daily for 2 minutes each (Vecsey et al., 2013).

EEG/EMG signals were sampled at 256 Hz and filtered at 0.5-30 Hz and 1-100 Hz, respectively, with 12A5 amplifiers (Astro-Med, West Warwick, RI). Data acquisition and visual scoring was performed using SleepSign software (Kissei Comtec, Inc. Japan). EEG/EMG recordings were scored in 4-second epochs as wake, non-rapid eye movement (NREM) sleep, or rapid eye movement (REM) sleep by a trained experimenter blind to experimental conditions.

Statistical analysis

Two-way repeated measures ANOVA was used to analyze behavior in the training sessions, with sleep condition as the independent factor, training session as the repeated measure factor, and exploration time of objects as the dependent variable. Two-way ANOVA was used to analyze spatial preference in the OPR task, with sleep condition and objects as factors and the delta percent exploration as the dependent variable. One-way ANOVA was used to analyze the average total exploration of objects in the test session of OPR, with sleep condition as the factor and exploration time during test session as the dependent variable. One-way ANOVAs were also used to analyze input output curves and to analyze the average of the last 20 minutes of fEPSP slopes from potentiated hippocampal slices from the OPR + NSD group and the 2 OPR + SD

groups. A student's t-test was used to analyze the average of the last 20 minutes of fEPSP slopes from potentiated hippocampal slices from *non-trained* NSD and SD ZT 2-5 groups. Two-way repeated measures ANOVAs were used to analyze paired pulse facilitation, time spent in NREM and REM sleep, and gentle handling during SD. Tukey's multiple comparison was selected for post hoc analyses for OPR and gentle handling. Dunnett's multiple comparison was selected for *post hoc* analyses of hippocampal synaptic plasticity. All statistical analyses were performed using SAS 9.3 software (SAS Institute Inc., Cary, NC). A probability level of $p < 0.05$ was considered significant. Data are presented as mean ± SEM.

Results

Immediate 4h and 6h sleep deprivation after object place recognition training impairs long-term memory

We first examined two periods of immediate SD during consolidation of spatial memory previously found to be sufficient in impairing hippocampus-dependent memory. We subjected separate groups of mice to either a 6 hour-period of SD (SD ZT 1-7) or a smaller 4 hour-period of SD (SD ZT 1-5) as outlined in **Fig. 2.1. A**. We pooled the NSD control groups from both SD experiments as this did not change the statistical outcome. The NSD and SD groups acquired the task at similar rates, measured by time spent exploring objects during each session (**Fig. 2.2. A**). A two-way repeated measures ANOVA revealed that the three groups did not differ in exploration times ($F_{(1,192)} = 0.0$, $p = 0.99$). All groups gradually reduced total exploration time ($F_{(2,60)} = 94.2$, $p < 0.05$). No interaction existed for session and group for exploration time ($F_{(4,72)} = 0.94$, $p = 0.39$). *Post-hoc* analysis revealed that exploration of the objects during the 2nd and 3rd training sessions were significantly lower that the 1st training session, indicating familiarization

with the objects over time (NSD 1st session compared to 2nd session: $t_{(192)} = 8.10$, $p < 0.05$; NSD 1st session compared to 3rd session: $t_{(192)} = 10.06$, $p < 0.05$) (SD 1st session compared to 2nd session: $t_{(192)} = 6.83$, $p < 0.05$; SD 1st session compared to 3rd session: $t_{(192)} = 8.16$, $p < 0.05$). Two way ANOVA revealed that sleep deprivation had an effect on the extent of change in preference for the displaced object between the 3rd training session and the Test session (delta preference) ($F_{(2,29)} = 12.41$, $p < 0.05$). There was a significant main effect of type of object (non-displaced object [NDO] vs. displaced object [DO]) in delta preference of object exploration ($F_{(1,29)} = 5.289$, $p < 0.05$). There was a significant interaction between object and sleep group ($F_{(2,29)} = 13.41$, $p < 0.05$). *Post hoc* analysis revealed that the NSD group exhibited enhanced preference for the DO during the test session relative to the NDO (NDO = -7.82 ± 1.54%, DO = 16.75 ± 2.69%; $p < 0.05$). Analysis of the immediate 6 hour SD group (**Fig. 2.2. B**) revealed that preference for the DO was not significantly different compared to the NDO (NDO = -0.75 ± 2.64%; DO = -3.88 ± 2.20%; $p = 0.99$). There was significantly decreased exploration of the DO in the immediate 6 hour SD group compared to the NSD group (NSD = 16.75 ± 2.69%, SD ZT 1-7 = -3.88 ± 2.20%; $p < 0.05$). Analysis of the immediate 4 hour SD group (**Fig. 2.2. B**) revealed that preference for the DO was not significantly different compared to the NDO (NDO = 1.03 ± 2.16%; DO = -0.47 ± 4.00%; $p = 0.99$). There was significantly decreased exploration of the DO in the immediate 4 hour SD group compared to the NSD group (NSD = 16.75 ± 2.69%, SD ZT 1-5 = -0.47 ± 4.00%; $p < 0.05$). Thus, our data illustrate, in agreement with existing literature, that hippocampus-dependent long-term memory is sensitive to 4 or more hours of sleep deprivation immediately following acquisition.

Delayed sleep deprivation during hours ZT 2-5 after object place recognition training impairs long-term memory

We next examined the impact of two periods of SD during the consolidation of spatial memory. We subjected separate groups of mice to one of two overlapping 3-hour SD periods during the first 4 hours of consolidation. We chose to examine these overlapping sleep deprivation time points because we had already discovered that 4 hours of immediate sleep deprivation (ZT 1-5) was sufficient to impair memory compared to the NSD control group. This observation suggests that the critical time window necessary for sleep existed within the first 4 hours of after learning in mice. Mice were sleep deprived for 3 hours immediately after training (ZT 1-4; immediate SD) or for 3 hours beginning an hour after training (ZT 2-5; delayed SD), as outlined in **Fig. 2.1. A**. The three groups (NSD, immediate SD, and delayed SD) acquired the task at similar rates, measured by time spent exploring objects during each session (**Fig. 2.3. A**). A two-way repeated measures ANOVA revealed that the three groups did not differ in exploration times ($F_{(2,36)} = 0.32$, $p = 0.73$). All groups gradually reduced total exploration time ($F_{(2,72)} = 199.88$, $p < 0.05$). No interaction existed for session and group for exploration time ($F_{(4,72)} = 1.85$, $p = 0.13$). *Post-hoc* analysis revealed that exploration of the objects during the 2nd and 3rd training sessions were significantly lower that the 1st training session, indicating familiarization with the objects over time (NSD 1st session compared to 2nd session: $t_{(72)} = 6.52$, $p < 0.05$; NSD 1st session compared to 3rd session: $t_{(72)} = 8.88$, $p < 0.05$) (SD ZT 1-4 1st session compared to 2nd session: $t_{(72)} = 5.2$, $p < 0.05$; SD ZT 1-4 1st session compared to 3rd session: $t_{(72)} = 9.06$, $p < 0.05$) (SD ZT 2-5 1st session compared to 2nd session: $t_{(72)} = 4.97$, $p < 0.05$; SD ZT 2-5 1st session compared to 3rd session: $t_{(72)} = 8.91$, $p < 0.05$). Two-way ANOVA revealed that SD group had an effect on the extent of change in preference for the displaced object between the 3rd

training session and the Test session (delta preference) ($F_{(2,36)}$ = 3.88, p < 0.05). There was a significant main effect of type of object (non-displaced object [NDO] vs. displaced object [DO]) in delta preference of object exploration ($F_{(1,36)}$ = 41.59, p < 0.05). There was a significant interaction between sleep group and type of object ($F_{(2,36)}$ = 3.24, p < 0.05). *Post hoc* analysis revealed that the NSD group exhibited enhanced preference for the DO during the test session relative to the NDO (NDO = -6.54 ± 1.24%,DO = 14.2 ± 1.99%; p < 0.05). The immediate SD group exhibited increased exploration of the DO compared to the NDO (NDO = -4.0 ± 3.78%, DO = 11.52 ± 3.96%; p < 0.05). There was no difference in exploration of the NDO between the NSD group and the immediate SD group (NSD = -6.54 ± 1.13%, SD ZT 1-4 = -4.0 ± 3.78%; p = 0.97). Similarly, there was no difference in the preference for the DO in the immediate SD group compared to the control NSD group (NSD = 14.2 ± 1.99%, SD ZT 1-4 = 11.52 ± 3.96%; p = 0.96) (**Fig. 2.3. B**). Analysis of the delayed SD group (**Fig. 2.3. B**) revealed that preference for the DO was not significantly different compared to the NDO (NDO = -4.82 ± 1.63%; DO = 2.38 ± 2.82%, p = 0.54). There was significantly decreased exploration of the DO in the delayed SD group compared to the NSD group (NSD = 14.2 ± 1.99%; SD ZT 2-5 = 2.38 ± 2.82%, p < 0.05). *Post hoc* analysis revealed that the exploration of the NDO was not significantly different in the delayed SD group compared to NSD controls (NSD = -6.54 ± 1.24%; SD ZT 2-5 = -4.82 ± 1.63%, p = 0.99). Thus, our data indicate that there is a specific time window, during ZT 2-5, where object-place memory consolidation is vulnerable to sleep deprivation.

Delayed sleep deprivation during hours ZT 3-5 and ZT 4-5 after object place recognition does not impair long-term memory

We wanted to determine whether the effects of sleep deprivation would still impair memory if constricted within the delayed 3 hour period already characterized as devastating to long-term memory. We sleep deprived separate groups of mice for 2 hours from ZT 3-5 and 1 hour from ZT 4-5 after OPR training, as outlined in **Fig. 2.1. A**. The NSD and SD groups acquired the task at similar rates, measured by time spent exploring objects during each session (**Fig. 2.4. A**). A two-way repeated measures ANOVA revealed that the three groups did not differ in exploration times ($F_{(1,244)} = 0.55$, $p = 0.46$). All groups gradually reduced total exploration time ($F_{(2,76)} = 225.2$, $p < 0.05$). No interaction existed for session and group for exploration time ($F_{(2,244)} = 0.72$, $p = 0.49$). *Post-hoc* analysis revealed that exploration of the objects during the 2nd and 3rd training sessions were significantly lower that the 1st training session, indicating familiarization with the objects over time (NSD 1st session compared to 2nd session: $t_{(244)} = 10.95$, $p < 0.05$; NSD 1st session compared to 3rd session: $t_{(244)} = 15.56$, $p < 0.05$) (SD 1st session compared to 2nd session: $t_{(244)} = 8.52$, $p < 0.05$; SD 1st session compared to 3rd session: $t_{(244)} = 14.03$, $p < 0.05$). Two-way ANOVA revealed that SD group did not have an effect on the extent of change in preference for the displaced object between the 3rd training session and the Test session (delta preference) ($F_{(2,37)} = 0.25$, $p < 0.78$). There was a significant main effect of type of object (non-displaced object [NDO] vs. displaced object [DO]) in delta preference of object exploration ($F_{(1,37)} = 53.07$, $p < 0.05$). There was not a significant interaction between sleep group and type of object ($F_{(2,37)} = 2.35$, $p = 0.10$) (**Fig. 2.4. B**). Although a trend existed for sleep group to affect exploration based on the type of object, this did not reach significance.

Sleep deprivation from ZT 1-4 or ZT 2-5 does not impair ability to explore objects during the test session

We found that ZT 2-5 was a critical time window during which sleep deprivation could disrupt long-term memory. Our measure of memory is the change in percent exploration of the objects during the test session compared to the last training session in the OPR task. However, sleep deprivation could also reduce general exploration of objects during the test session, which is an effect unrelated to memory. Because sleep deprivation could potential reduce exploration during the test session, we decided to compare the average of total time spent exploring all the objects during the test session between the NSD, SD ZT 1-4 and SD ZT 2-5 groups. A one-way ANOVA revealed that sleep deprivation did not have an effect on the total exploration of all objects during the test session ($F_{(2,39)}$ = 0.47, p = 0.68) (**Fig. 2.5.**).

Delayed sleep deprivation during hours ZT 2-5 after object-place recognition training disrupts LTP

Due to the distinct behavioral outcomes that resulted from the immediate and delayed 3 hour periods of sleep deprivation, we examined hippocampal synaptic function and L-LTP in area CA1 after OPR training followed by these 2 time periods of sleep deprivation. Input/output curves did not differ between NSD, immediate SD, and delayed SD groups (**Fig. 2.6. B**; $F_{(2,20)}$ = 2.96, p = 0.08). Paired pulse facilitation (PPF) was not altered in either of the SD groups compared to the NSD group after training (**Fig. 2.6. A**; $F_{(8,100)}$ = 1.38, p = 0.21). Synaptic strength, measured as the maximum fEPSP slope induced by stimulation, was comparable between groups (OPR + NSD = 7.2 + 0.42 -mV/ms; OPR + SD ZT 1-4 = 7.4 + 0.60 -mV/ms; OPR + SD ZT 2-5 = 7.0 + 0.39 -mV/ms; $F_{(2,36)}$ = 0.13, p = 0.88). We compared L-LTP from both the delayed and immediate SD groups to a NSD control group. A one-way ANOVA on the average of the last 20 minutes of recordings in each group revealed that sleep group had an effect on L-LTP,

$F_{(2,16)}$ = 4.85, p < 0.05. *Post hoc* analysis revealed that L-LTP was elicited in slices from the immediate SD group, the potentiation was not significantly different compared to slices from NSD control group (**Fig. 2.6. C** and **Fig. 2.6. D**; OPR + NSD = 159% ± 14.33%, OPR + SD ZT 1-4 = 175% ± 19.67%; p = 0.71). Conversely, the delayed SD group displayed impaired LTP compared to the NSD control group and the immediate SD group (OPR + SD ZT 2-5 = 109% ± 10.50%; p < 0.05). We also examined the effect of sleep deprivation on LTP during the ZT 2-5 time window without prior behavioral training. A student's t-test on the averaged fEPSP slope of the last 20 minutes of recordings in the SD ZT 2-5 group compared to the NSD group revealed that 3 hours of sleep deprivation without prior training did not affect LTP ($t_{(12)}$ = -1.09, p = 0.30) (**Fig. 2.6. E** and **2.6. F**). This pinpoints a specific 3-hour time window *after* learning where sleep deprivation during consolidation disrupts not only memory, but hippocampal LTP as well.

Mice spent similar time in NREM and REM sleep throughout the period ZT 1-5

Following these results, we wanted to examine whether sleep differed during these two periods and thereby, could potentially account for the difference in the effect of sleep deprivation on memory and plasticity. We measured NREM and REM sleep in mice from ZT 1-5 using polysomnography. We compared the time spent in NREM and REM each hour from ZT1 to ZT 5 (NREM sleep in minutes: ZT 1-2 = 29.49 ± 4.49, ZT 2-3 = 38.44 ± 1.74, ZT 3-4 = 34.16 ± 3.26, ZT 4-5 = 31.69 ± 2.06; REM sleep in minutes: ZT 1-2 = 6.26 ± 1.45, ZT 2-3 = 6.03 ± 1.12, ZT 3-4 = 5.76 ± 0.78, ZT 4-5 = 5.43 ± 0.61). We found no difference in time spent in NREM or REM sleep from ZT1 to ZT 5 (**Fig. 2.7. A**; $F_{(3,20)}$ = 1.090; p = 0.3763).

Increased gentle handling was required to maintain wakefulness in the delayed sleep deprivation group in comparison to the immediate sleep deprivation group

To determine if the amount of stimulation needed to keep animals awake differed between the two SD periods, the frequency of manipulation during each hour of sleep deprivation was analyzed. For cage taps (**Fig. 2.8. A**), there was a significant treatment group by hour interaction ($F_{(2,18)} = 5.67$, $p < 0.05$). *Post hoc* analyses indicated that the number of cage taps to maintain the wake state was significantly greater in the first hour for the delayed group compared to the first hour in the immediate SD group (OPR + SD ZT 1-4 = 4.8 ± 0.55, OPR + SD ZT 2-5 = 11.83 ± 1.48; $p < 0.05$). The frequency of cage shaking was also measured across the two 3-hour SD groups (**Fig. 2.8. B**). A two-way repeated measures ANOVA revealed an interaction between hour and treatment group ($F_{(2,18)} = 10.19$; $p < 0.05$). *Post hoc* analyses revealed that compared to the immediate SD group, animals from delayed SD group required more cage shakes to sustain wakefulness during the first hour of the 3-hour SD (OPR + SD ZT 1-4 = 4.4 ± 0.84, OPR + SD ZT 2-5 = 12.17 ± 1.48; $p < 0.05$). Our findings indicate that the immediate SD group required less gentle handling manipulation to maintain wake than the delayed sleep deprivation group within the 1st hour.

Discussion

Our study investigated whether there is a critical time window for sleep deprivation during memory consolidation to impair hippocampus-dependent long-term memory and plasticity. Similar to prior findings, an immediate 4 hours and 6 hours of SD impaired hippocampus dependent long-term memory. We found that as little as 3 hours of SD following learning impairs spatial memory in mice. More importantly, we observed that the timing of sleep loss is critical. When the onset of SD was delayed for an hour after learning, hippocampus-dependent memory consolidation was impaired compared to when mice were sleep deprived beginning immediately after learning (**Fig. 2.3. B**).

These results were not due to SD causing a decrease in total exploration of objects during test session. First, we account for that by using a metric where we compare the percent exploration in the test session to the last training session. Second, we did not observe reduced total exploration of objects during the test session in SD compared to the NSD group (**Fig. 2.5.**). We also found, to the best of our knowledge, the first evidence that SD after learning impaired hippocampal plasticity, but only when delayed by one hour following training (**Fig. 2.6. D**). Thus memory consolidation and synaptic plasticity exhibit a similar sensitivity to sleep deprivation.

After determining that the delayed 3-hour SD period is sufficient for the manifestation of memory and plasticity impairments, we examined potential factors that could explain these 2 different 3-hour periods of sleep deprivation produced distinct outcomes. First, it was possible that there are qualitative differences in the type of sleep engaged in between these two periods. Previous findings have associated periods of more NREM sleep with stronger consolidation of hippocampus-dependent memories (Plihal & Born, 1997; van der Helm, Gujar, Nishida, & Walker, 2011; Wamsley, Tucker, Payne, & Stickgold, 2010). If animals were sleep deprived during a time characterized by more NREM sleep, this could possibly explain our findings. We found that mice exhibited similar NREM and REM sleep across the hours ZT 1-5 and during the ZT 1-4 and ZT 2-5 periods (**Fig. 2.7. A** and **2.7. B**). This suggests that other factors must be at work in producing our main finding.

On the other hand, although naïve animals did not show differences in sleep patterns throughout ZT 1-5 (**Fig. 2.7. A**), modulation in sleep due to prior training could affect the amount of sleep lost in the delayed SD condition compared to the sleep lost in immediate SD condition. Previous studies have indicated that training in open field, novelty exposure, as well as handling leads to an immediate decrease in sleep followed

by increases in later periods of sleep (Tang et al., 2004, 2005a, 2007; Hellman and Abel, 2007). Other studies, however, showed an increase in REM sleep following learning in the water maze task and inhibitory avoidance task (Smith, 1985b; Smith and Rose, 1996). The present study supports these findings of altered sleep patterns after training. Our assessment through measurement of gentle handling showed that the immediate SD group required less manipulation to maintain wake than the delayed sleep deprivation group within the first hour, lending further support to previous findings that training changes sleep patterns in animals (**Fig. 2.8. A** and **2.8. B**). The delayed SD group required more manipulation throughout the SD procedure, which could be a result of increased sleep pressure in the delayed group. We think that memory deficits observed in the delayed SD group could be attributed to a combination of sleep deprivation and sleep pressure rather than the increased frequency of stimulation during SD. In line with our findings, a previous study from Hagewoud and colleagues (2010) also showed that impaired memory was a result of extent of sleep loss and not due to frequency of stimulation needed to keep animals awake (Hagewoud et al., 2010c). Furthermore, our work suggests that delayed SD is potentially more disruptive than immediate SD due to prior training affecting later sleep, therefore enhancing the impact of sleep deprivation during the later time period. This may explain why the delayed SD without prior behavioral training had no effect on LTP (**Fig. 2.6. E** and **2.6. F**), while prior training followed by delayed SD impaired LTP (**Fig. 2.6. C** and **2.6. D**). More extensive examination is needed in future, including recording sleep after OPR learning and measuring slow wave activity over the hours following OPR training. Other groups have used various instrumentation techniques to record from the cortex, as well as the hippocampus, in experiments that involved another object recognition task, novel object

recognition, so this may be a future area of interest (Clarke et al., 2010; Rolls et al., 2011).

Another potential reason that delayed SD would impair the consolidation of object-place memory is that this delayed period of SD may disrupt molecular signaling pathways known to be important for memory consolidation. For instance, SD has previously been shown to reduce levels of the second messenger, cAMP in the hippocampus (Vecsey et al., 2009). cAMP signaling in the hippocampus has been observed to peak at 3 hours after acquisition, along with α-amino-3-hydroxy-5-methyl-isoxazolepropionate (AMPA) receptor and dopamine (D1) receptor function (Bernabeu et al., 1997a, 1997b). Also, cAMP signaling in the hippocampus has been observed to oscillate in a circadian fashion with increases from ZT 4 through ZT 8 (Eckel-Mahan et al., 2008). Luo and Colleagues showed that this period of increased cAMP levels occurred during REM sleep, accompanied by increases in mitogen activated protein kinase (MAPK) and cAMP-response element binding protein (CREB) levels (Luo et al., 2013). Because our delayed SD manipulation occurs during the period of increased signaling necessary for memory consolidation, it is likely preventing consolidation by disrupting these active signaling cascades. Thus, the memory and hippocampal plasticity deficits that were observed in the delayed SD group could be attributed to disrupted activation of the molecular signaling pathways necessary for memory consolidation, which selectively occurs in the later time period. Future experiments will be needed to explore these ideas.

Impairment in the maintenance of hippocampal LTP due to SD has been observed in freely moving animals (Romcy-Pereira and Pavlides, 2004; Ishikawa et al., 2006) and in hippocampal slices prepared from sleep-deprived animals (Campbell et al., 2002; McDermott et al., 2003; Kopp et al., 2006; Vecsey et al., 2009). These LTP

experiments examined SD without prior behavioral training, which more accurately reflects the effects of SD on acquisition of memory. Indeed, it has not previously been shown that SD during consolidation can disrupt LTP. The current study is the first examination of LTP susceptibility to SD after training in a learning task, during consolidation. Our finding that similar SD temporal parameters impair memory and disrupt LTP strengthens the conclusion that sleep is required during this particular time period for memory storage.

We were concerned that prior training would affect LTP induction because a few studies have previously reported alterations in plasticity, in some cases de-potentiation of LTP, after exploration of a novel environment with objects (Kemp & Manahan-Vaughan, 2004; Xu, Anwyl, & Rowan, 1998). We did not observe this de-potentiation of LTP after training in our NSD group. This could be due to reintroduction to the environment and objects over multiple sessions in our paradigm, thereby decreasing the novelty aspect over time. Induction of LTP after introduction to a novel environment has been shown to either enhance LTP induced by weak stimulation or not affect LTP induced by strong stimulation (Li et al., 2003; Dong et al., 2012). We do not see an effect of prior training on cAMP-dependent LTP induced in slices obtained from NSD animals, possibly due to our use of a strong stimulation protocol. Compared to our prior experiences eliciting LTP in slices obtained from non-trained NSD animals, the LTP elicited from prior trained animals did not appear enhanced or reduced. Therefore, our LTP findings after training + SD are comparable to our behavioral SD findings because prior exposure to a novel stimulus does not affect resulting LTP in our experiments.

We also explored the effects of shorter periods of SD on memory consolidation within this critical ZT 2-5 period; a 1 hour SD from ZT 4-5 and a 2 hour SD from ZT 3-5 after training. Although we did not observe a significant decline in percent exploration in

either SD groups compared to the NSD group, we did see a trend of decreasing exploration of the displaced in object in both SD groups. However, these periods of SD were not sufficient to impair memory. This suggests that the impairment of long-term memory is a result of timing combined with duration of SD. Prior work has focused solely on the duration of SD. Current studies are now beginning to explore the relationship between timing and duration of sleep in enabling memory consolidation (Genzel et al., 2013). Examining combined aspects of SD, such as duration and timing, on memory provides an exciting avenue for exploration in future studies.

Abnormalities in sleep are accompanied by decreased vigilance, metabolic alterations, and psychiatric disorders (Benca et al., 1992; Spiegel et al., 1999; Stickgold et al., 2000). Cognitive deficits are often cited as a result of sleep loss. We have found a narrow critical period during consolidation that is sensitive to the effects of SD. Our finding that the delayed SD post training, rather than the immediate SD post training, impairs both memory and LTP suggests that consolidation processes during the first hour after training are not dependent on sleep. Studies have observed an increase in hippocampal sharp-wave ripples (SPW-R) in the first period of slow wave sleep (Kudrimoti et al., 1999; Ramadan et al., 2009) critical for memory consolidation and plasticity (Born and Feld, 2012; Chauvette et al., 2012; Grosmark et al., 2012). While our findings do not explicitly support this hypothesis considering that the absence of the first period of slow wave sleep, including the accompanying hippocampal SPW-R, does not lead to deficits in memory consolidation, our study does not examine SPW-R. Thus, we cannot speak to the importance of this first period SPW-R to sleep-dependent memory consolidation, but future study is warranted.

A potential focus of future research could be exploration of this time window to determine what is being disrupted on a molecular level. Recent research has already

begun to examine molecular disruption that occurs with minimal SD, noting disruption in hippocampal cAMP, increased phosphodiesterase 4 (PDE4) enzyme activity in the hippocampus, as well as altered transcription and translation in the hippocampus with only 5 hours of SD (Vecsey et al., 2009, 2012). However, little is known regarding molecular changes occurring specifically after SD during the consolidation period. Our main findings highlight a time window during which we can begin to explore disruptions in signaling resulting from SD during consolidation, potentially expanding our understanding of molecular mechanisms relevant to both sleep and memory. The continued examination of sleep deprivation and sleep/wake states during consolidation will enable a better understanding of the effects of both sleep and sleep loss on cognition and memory, informing developers of potential novel therapeutic treatments.

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Author Contributions

Experiments were conceived and designed by Toni-Moi Prince, Dr. Mathieu Wimmer, Dr. Jennifer Choi, Dr. Robbert Havekes, Dr. Sara Aton, and Dr. Ted Abel. Electrophysiological and behavioral experiments were performed by Toni-Moi Prince. Polysomnography data was collected by Dr. Mathieu Wimmer. This chapter was written by Toni-Moi Prince with comments and editing by Dr. Jennifer Choi and Dr. Ted Abel.

FIGURE 2.1.

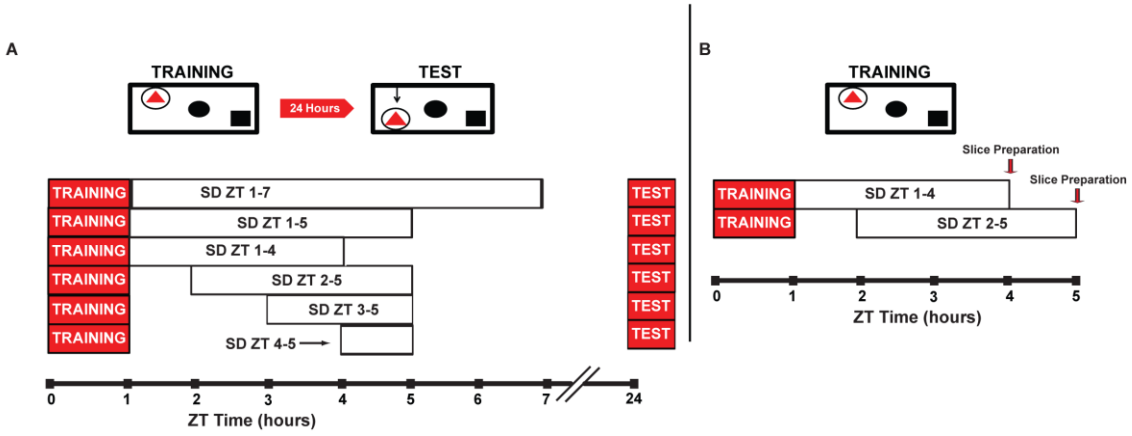

Figure 2.1. *Schematic depicting the behavioral and LTP experimental design.*

(A) Behavioral experimental design: The top diagram depicts the OPR task used to examine hippocampus-dependent memory. The training sessions include repeated exposure to 3 distinct objects and began at lights on ZT 0. The test session occurred 24 hours following training. The bottom diagram depicts the post-training sleep deprivation time periods for each behavioral experiment. After the last training session, mice were subjected to varying hours of sleep deprivation to assess the specific time window for sleep deprivation to impair memory. (B) LTP experimental design: The top diagram demonstrates that mice were subjected to the same training as those that were in the behavioral experiment assessing memory. However, there was no later test period for these animals. The bottom diagram depicts the sleep deprivation periods after training as well as when hippocampal slices were collected for field recordings. Recordings obtained from NSD control groups were later pooled, and LTP from the sleep deprivation groups were compared to this pooled group. Prior to pooling the data from the NSD control groups, we determined that the NSD control groups were not significantly different from each other.

FIGURE 2.2.

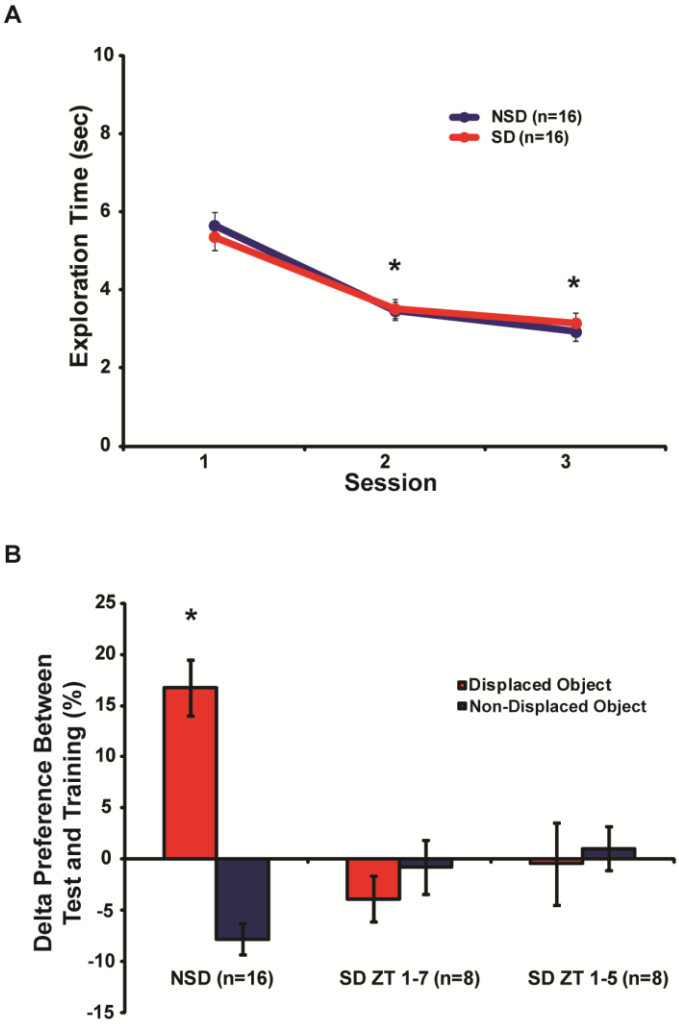

Figure 2.2. *4 hours and 6 hours of immediate sleep deprivation impairs spatial memory.*

(A) Mean exploration time of objects during each session was not significantly different between the NSD and SD experimental groups ($F_{(2,192)}$ = 0.94, p = 0.39). Mean exploration time gradually decreased during training ($F_{(2,60)}$ = 94.2, p < 0.05) (blue = NSD, red = SD). (B) Mean delta percentage of exploration of NDO and DO between test session and final training session for SD and NSD groups. Mean delta percentage of DO exploration significantly increased for the NSD group (n = 16) compared to their exploration of the NDO ($F_{(2,29)}$ = 13.41, p < 0.05; $t_{(29)}$ = 6.69, p < 0.05). Mean delta percentage of DO exploration did not significantly change compared to exploration of the NDO for animals subjected to immediate 6 hours of SD (SD ZT 1-7, n = 8) ($F_{(2,29)}$ = 13.41, p < 0.05; $t_{(29)}$ = -0.60, p = 0.99). For mice subjected to an immediate 4 hours of SD (SD ZT 1-5, n = 8), Mean delta percentage of DO exploration was not significantly different from percentage exploration of the NDO ($F_{(2,29)}$ = 13.41, p < 0.05; $t_{(29)}$ = -0.29, p = 0.99). Error bars indicate ± SEM.

FIGURE 2.3.

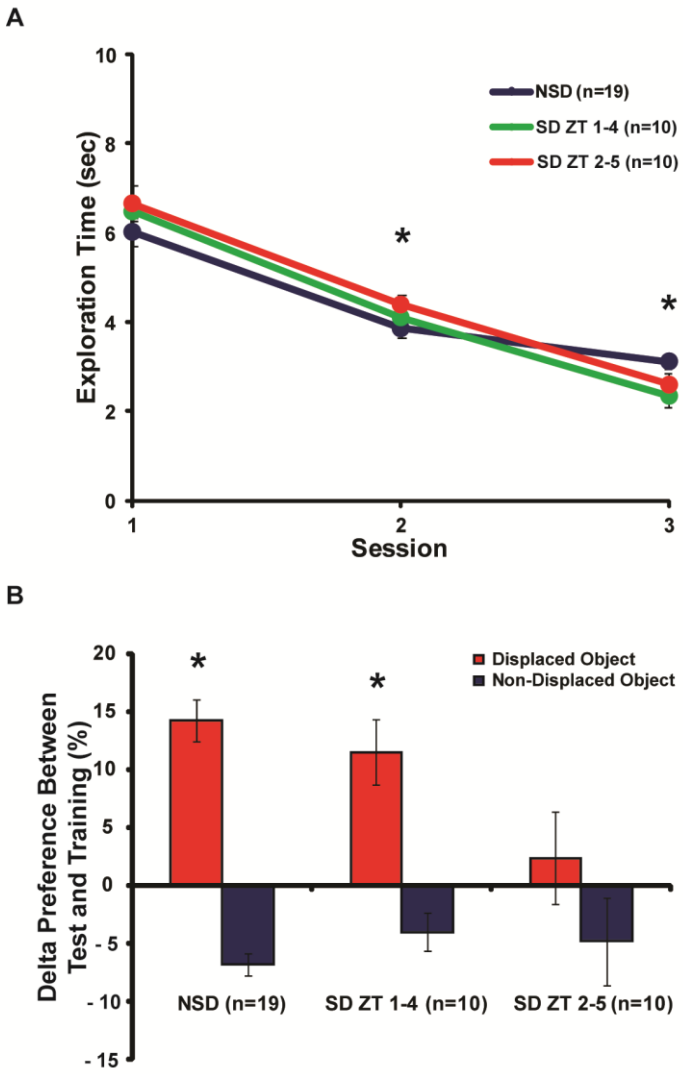

Figure 2.3. *A specific 3-hour sleep deprivation period impairs spatial memory.*

(A) Mean exploration time of objects during each session was not significantly different between the NSD and the two SD experimental groups ($F_{(4,72)}$ = 1.85, p = 0.13). Mean exploration time gradually decreased during training ($F_{(2,72)}$ = 199.88, p < 0.05) (blue = NSD, red = SD). (B) Mean delta percentage of exploration of NDO and DO between test session and final training session for SD and NSD groups. Mean delta percentage of DO exploration significantly increased for the NSD group (n = 19) compared to their exploration of the NDO ($F_{(2,36)}$ = 3.24, p < 0.05; $t_{(36)}$ = 6.75, p < 0.05). Mean delta percentage of DO exploration significantly increased for SD ZT 1-4 group (n = 10) compared to their exploration of the NDO ($F_{(2,36)}$ = 3.24, p < 0.05; $t_{(36)}$ = 3.66, p < 0.05). For mice subjected to a delayed SD from ZT 2-5 (n = 10), Mean delta percentage of DO exploration was not significantly different from percentage exploration of the NDO ($F_{(2,36)}$ = 3.24, p < 0.05; $t_{(36)}$ = 1.70, p = 0.53). Error bars indicate ± SEM.

FIGURE 2.4.

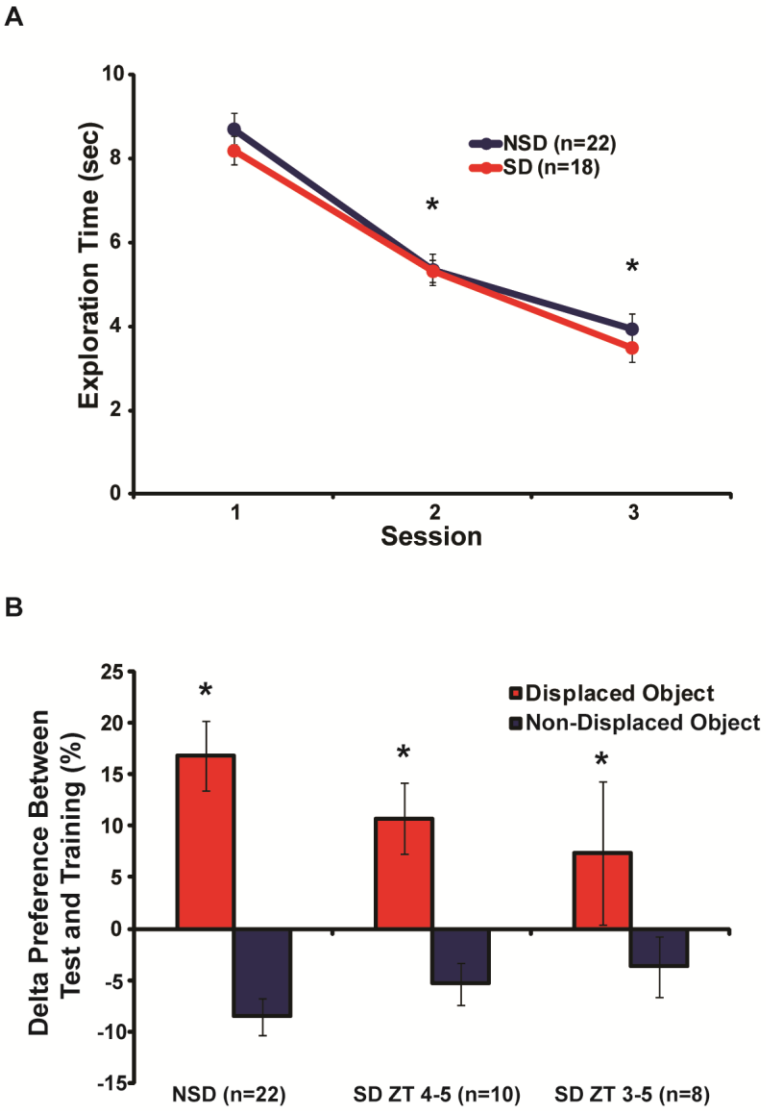

Figure 2.4. *A delayed 2-hour and 1-hour sleep deprivation period does not impair spatial memory.*

(A) Mean exploration time of objects during each session was not significantly different between the NSD and SD experimental groups ($F_{(2,244)}$ = 0.72, p = 0.49). Mean exploration time gradually decreased during training ($F_{(2,76)}$ = 225.2, p < 0.05) (blue = NSD, red = SD). (B) All groups (NSD, n = 22; SD ZT 3-5, n = 8; SD ZT 4-5, n = 10) successfully discriminated between the displaced and non-displaced object ($F_{(1,37)}$ = 53.07, p < 0.05), indicating that no effect of sleep group on type of object exploration existed ($F_{(2,37)}$ = 2.35, p = 0.10). Error bars indicate ± SEM.

FIGURE 2.5.

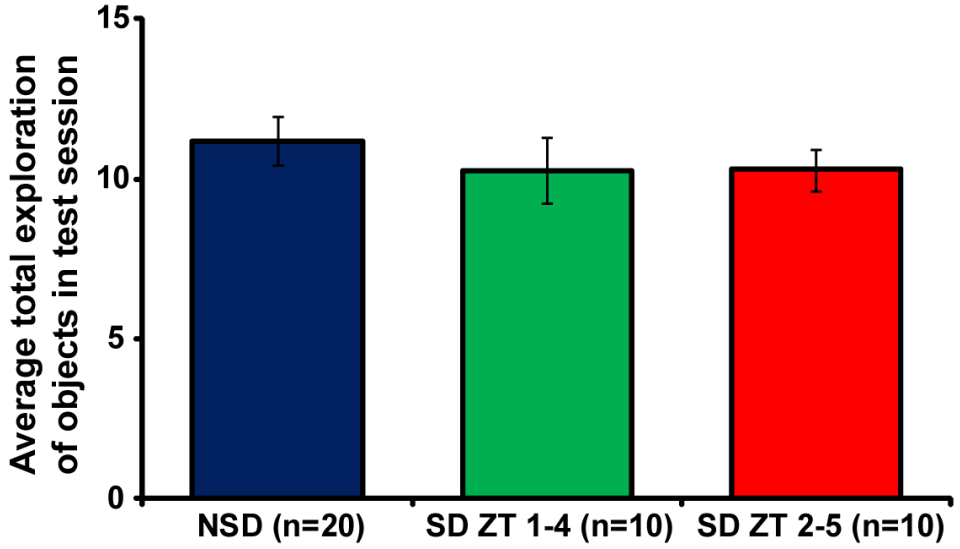

Figure 2.5. *3 hours of immediate or delayed sleep deprivation does not alter total exploration time of objects during test session.*

Mean exploration time of objects during test session was not significantly different between the NSD, SD ZT 1-4, and SD ZT 2-5 experimental groups ($F_{(2,39)}$ = 0.47, p = 0.68). (blue = NSD, green = SD ZT 1-4, red = SD ZT 1-5). Error bars indicate ± SEM.

FIGURE 2.6.

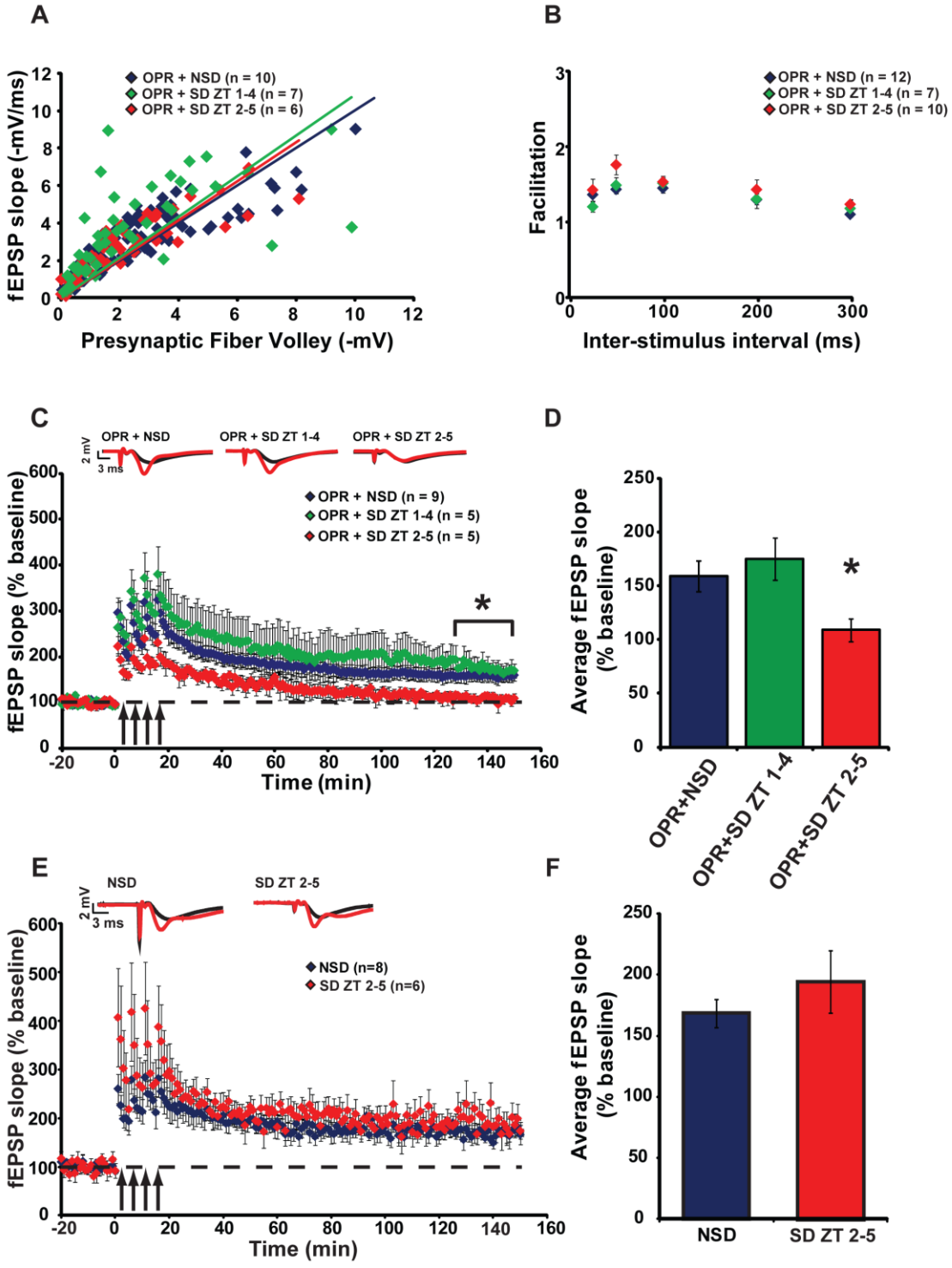
A
fEPSP slope (-mV/ms)
Presynaptic Fiber Volley (-mV)
OPR + NSD (n = 10)
OPR + SD ZT 1-4 (n = 7)
OPR + SD ZT 2-5 (n = 6)
B
Facilitation
Inter-stimulus interval (ms)
OPR + NSD (n = 12)
OPR + SD ZT 1-4 (n = 7)
OPR + SD ZT 2-5 (n = 10)
C
OPR + NSD
OPR + SD ZT 1-4
OPR + SD ZT 2-5
2 mV
3 ms
OPR + NSD (n = 9)
OPR + SD ZT 1-4 (n = 5)
OPR + SD ZT 2-5 (n = 5)
fEPSP slope (% baseline)
Time (min)
*
D
Average fEPSP slope (% baseline)
OPR+NSD
OPR+SD ZT 1-4
OPR+SD ZT 2-5
*
E
NSD
SD ZT 2-5
2 mV
3 ms
NSD (n=8)
SD ZT 2-5 (n=6)
fEPSP slope (% baseline)
Time (min)
F
Average fEPSP slope (% baseline)
NSD
SD ZT 2-5

Figure 2.6. *Delayed sleep deprivation after object-place recognition training disrupts LTP.*

(A) Input–output curves relating the amplitude of the presynaptic fiber volley to the initial slope of the corresponding fEPSP at various stimulus intensities were not altered in slices from either OPR trained SD groups compared to the OPR trained NSD group ($F_{(2,20)}$ = 2.96, p = 0.08). (B) Paired-pulse facilitation, a short-term form of synaptic plasticity, did not differ in slices obtained from either of the OPR trained SD groups compared to slices from the OPR trained NSD group ($F_{(8,100)}$ = 1.38, p = 0.21). (C) L-LTP induced by spaced 4-train stimulation was not impaired in slices from OPR trained animals in the NSD group (blue, n = 9). L-LTP induced by spaced 4-train stimulation was not impaired in slices from the OPR + SD ZT 1-4 group (green, n = 5). L-LTP induced by spaced 4-train stimulation was attenuated in slices from the OPR + SD ZT 2-5 group (red, n = 5) compared to the OPR + NSD group. (D) Bar graph depicts the average fEPSP slope over the final 20 minutes of recording for OPR + NSD, OPR + SD ZT 1-4, and OPR + SD ZT 2-5 group ($F_{(2,16)}$ = 4.85, $p < 0.05$). Post hoc tests showed that the SD ZT 2-5 group was significantly different from both NSD and SD 1-4 group ($p < 0.05$). (E) L-LTP induced by spaced 4-train stimulation was not impaired in slices from *non-trained* SD ZT 2-5 group compared (red, n = 6) to *non-trained* NSD control group (blue, n = 8). (F) Bar graph depicts the average fEPSP slope over the final 20 minutes of the recording for NSD and SD ZT 2-5 group ($t_{(12)}$ = -1.09, p = 0.30). In all sample sweeps, black traces indicate baseline, and red traces were acquired approximately 2 hours after stimulation. Calibration: 2 mV, 3 ms. Error bars indicate ± SEM.

FIGURE 2.7.

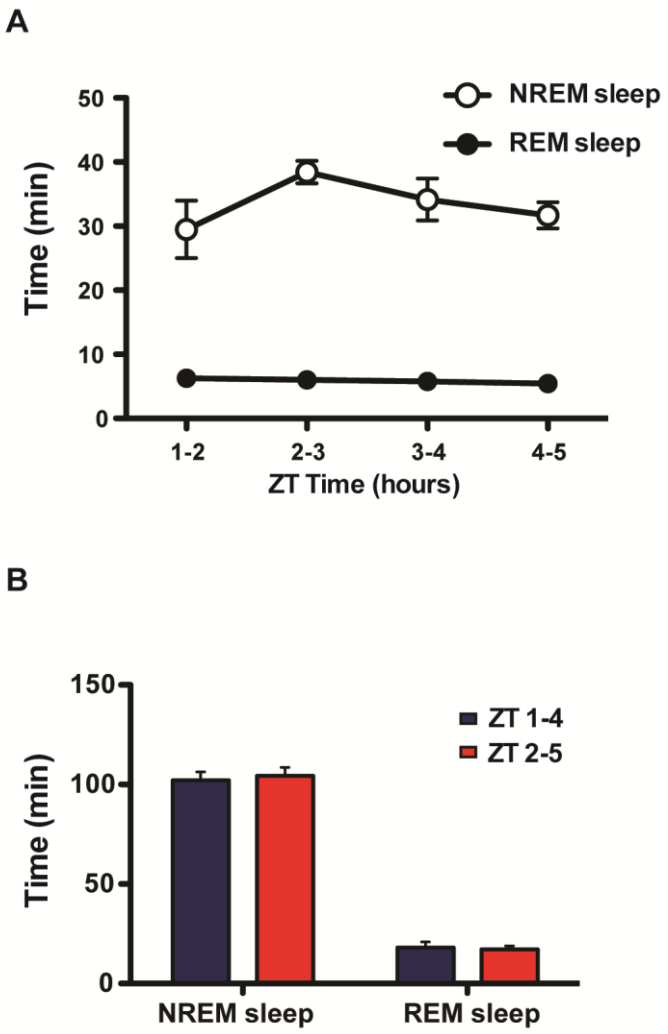

Figure 2.7. *Mice exhibit similar sleep patterns during periods ZT 1-5.*

(A) Mean time in minutes spent in NREM and REM sleep during each hour across ZT 1-5 is shown. Mice (n=6) spend comparable time in NREM sleep for each hour displayed. Mice also spend comparable time in REM sleep for each hour displayed $F_{(3,20)} = 1.090$; $p = 0.3763$. (B) Mean time in minutes spent in NREM sleep and REM sleep from ZT 1-4 and ZT 2-5 is shown. Mice (n = 6) spend comparable time in NREM sleep as well as REM sleep during these periods. Error bars indicate ± SEM.

FIGURE 2.8.

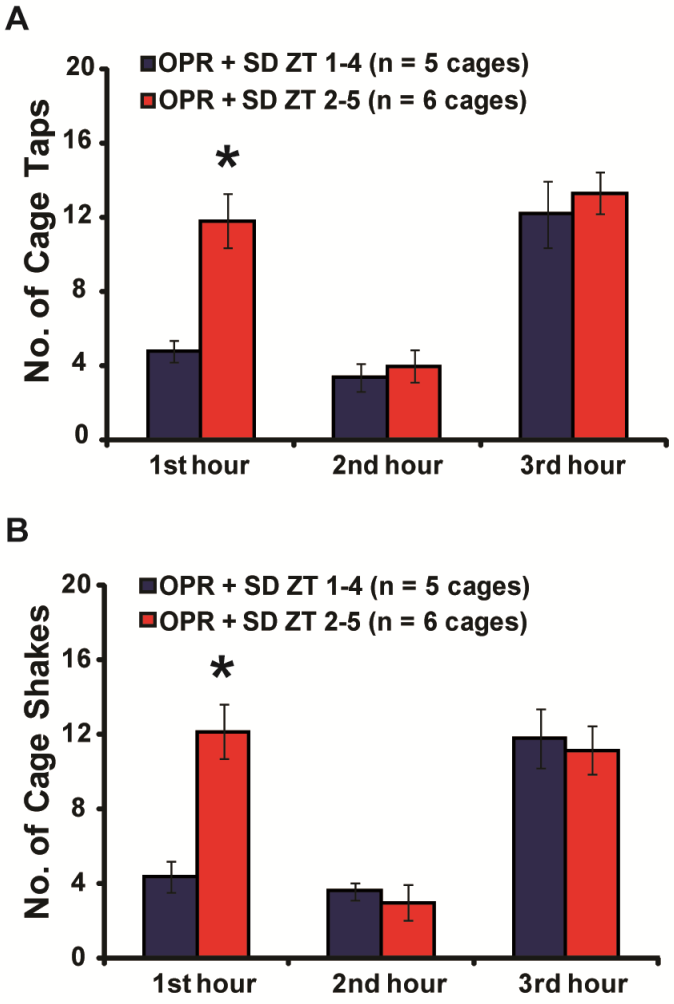

Figure 2.8. *The early sleep deprivation group requires less disturbance to achieve a wakeful state in the 1st hour of SD than the SD ZT 2-5 group.*

(A) Mean number of cage taps required to maintain wakefulness across 3 hours of SD after object-place recognition training. Frequency of cage taps in the 1st hour of sleep deprivation was significantly higher in the SD ZT 2-5 group (n = 6) than the SD ZT 1-4 group (n = 5) ($F_{(2,18)}$ = 5.67, $p < 0.05$; $t_{(18)}$= 4.60, $p < 0.05$). (B) Mean number of cage shakes required to maintain wakefulness across 3 hours of SD after object-place recognition training. Frequency of cage shakes in the 1st hour of SD was significantly higher in SD ZT 2-5 group than the SD ZT 1-4 group ($F_{(2,18)}$ = 10.19, $p < 0.05$; $t_{(18)}$ = 4.82, $p < 0.05$). Error bars indicate ± SEM.

CHAPTER THREE

PDE4A MEDIATES DISRUPTION OF HIPPOCAMPAL FUNCTION INDUCED BY SLEEP DEPRIVATION

Abstract

Insufficient sleep impairs hippocampal function, contributing to many cognitive disorders and psychiatric diseases. However, the molecular mechanisms by which sleep deprivation impairs hippocampal function are not well characterized. Previously, phosphodierasterase 4A5 (PDE4A5) has been implicated as an enzyme of interest for producing behavioral and synaptic plasticity deficits observed with sleep deprivation. Suppression of PDE4 rescued deficits in hippocampal function caused by sleep deprivation. Although inhibition of PDE4 rescues impaired hippocampal function in the face of sleep deprivation, it is still unknown whether this can be attributed to PDE4A, specifically. To determine the role of PDE4A in the impairment of hippocampal function caused by sleep loss, we examined the effect of loss of PDE4A on sleep-wake behavior, as well as memory and plasticity in the face of 5 hours of sleep loss. We found that loss of PDE4A modulated sleep during the active period, and rescued effects of sleep deprivation on spatial memory. These results suggest that PDE4A partially plays a role in sleep loss disruption in hippocampal function and may contribute to sleep-wake behavior exhibited by rodents. These results present a future potential therapeutic target in the fight against ensuing cognitive damage that commonly accompanies sleep loss.

Introduction

Insufficient sleep is a recurring affliction that unfortunately affects a substantial percentage of the population worldwide. Although not originally considered as a dire problem amongst the mainstream, chronic sleep deprivation for extended periods of time contributes to devastating consequences in both mental and physical health. One of the major consequences of sleep deprivation on the brain is impaired memory requiring the hippocampus for consolidation (Smith and Rose, 1997; Youngblood et al., 1997; Guan et al., 2004; Ruskin et al., 2004; Havekes et al., 2012; Abel et al., 2013; Prince and Abel, 2013). However, it is unclear what signaling pathways are being jeopardized as a result of sleep loss. Research to discover the mechanisms that lead to sleep deprivation-induced memory impairments is now being given precedence. One identified molecular target of sleep deprivation is the 2nd messenger cyclic adenosine monophosphate (cAMP), known to be important for memory consolidation (Abel and Lattal, 2001; Xia and Storm, 2012). cAMP has been shown to decrease after sleep deprivation, which affects the activity of its downstream targets such as protein kinase A (PKA), and the PKA targets; serine 845 of the AMPA receptor GluA1 subunit and serine 133 of cAMP response element-binding protein (CREB) (Vyazovskiy et al., 2008; Ravassard et al., 2009; Vecsey et al., 2009; Hagewoud et al., 2010a; Alhaider et al., 2011).

The reduction in hippocampal cAMP levels after a period of sleep deprivation is due to increased activity of phosphodiesterase 4 (PDE4). PDE4 belongs to the Class I PDE superfamily. The PDE4 group of enzymes in particular are responsible for the degradation of cyclic AMP in the hippocampus. Within the PDE4 family resides four genes: PDE4A, PDE4B, PDE4C and PDE4D. PDE4A is highly expressed in the CA1 region of the hippocampus, the area of study for many sleep deprivation effects on memory (Conti and Beavo, 2007). Our lab observed that sleep deprivation increases

PDE4A5 protein expression. Inhibition of PDE4 activity, via rolipram, prevents sleep deprivation from impairing memory consolidation as well as cAMP dependent forms of synaptic plasticity within the hippocampus (Vecsey et al., 2009). This suggests that the effects of sleep deprivation are modulated by PDE4 activity. Because rolipram inhibits all 4 genes in the PDE4 family, the rescue effects could be due to any of the 4 genes in the family. These results suggest that the PDE4 family of genes plays an important role in sleep deprivation consequences on memory and plasticity. However, since PDE4A5 was up-regulated with sleep deprivation, PDE4A became a prime target for exploration. Examination of loss of PDE4A has yet to be conducted in the effects of sleep deprivation on memory. Here, we examine whether constituitive deletion of PDE4A can rescue memory in spite of sleep deprivation.

Materials and Methods

Mice

One hundred adult male mice (2 to 4 months of age) were single housed and kept on a 12h/12h light/dark schedule with lights on at 7:00 AM (ZT 0). The generation of phosphodiesterase 4a, PDE4A, constitutive knockout mice ($PDE4A^{-/-}$) has previously been described (Jin, 1999; Jin and Conti, 2002; Jin et al., 2005). These animals were on a C57Bl/6 background (backcrossed for at least 10 generations). WT littermates were used as control in all experiments. Genotype was determined by PCR of genomic DNA. Food and water were available *ad libitum* throughout the experiments. All experiments were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania and were carried out in accordance with all National Institutes of Health guidelines.

Sleep Deprivation

To assess the effects of sleep deprivation (SD) on memory, mice (n = 39) were sleep-deprived for 5 hours using the gentle handling technique involving manual cage tapping, cage shaking, nestlet disturbance, and gentle animal prodding (Ledoux et al., 1996; Vecsey et al., 2013). For behavioral tasks, sleep deprivation was administered immediately after task training.

Behavior

Activity Monitoring

Activity was monitored using an infrared beam-break based system (Opto M3, Columbus Instruments, Columbus, OH), which provided a high resolution grid covering the horizontal plane. The beams were spaced 0.5 inches apart and counts of beam breaks were compiled every 10 seconds. In our analysis methodology, mice (n = 15) were considered asleep, if they did not break a single beam in the horizontal plane for 40 seconds or longer. We chose this threshold because it was validated in a previous study using the same activity monitoring system (Pack et al., 2007).

Contextual Fear Conditioning (CFC)

Fear conditioning experiments were performed in chambers using the methods previously described (Vecsey et al., 2009). Mice (n = 13) were handled for 3 consecutive days for 1 minute each day. For CFC, mice were placed into the conditioning chamber and received a 2 second 1.5 mA scrambled footshock 2.5 minutes after placement into the chamber. Mice were removed from the chamber after 3 additional minutes. The test session occurred 24 hours after conditioning during which mice were reexposed to the context for 5 minutes. Freezing behavior, the complete absence of movement during the test session, indicates the strength of the memory (Fanselow, 1980). The behavior of

each mouse was sampled at 5-second intervals and the percentage of those intervals in which the mouse froze was calculated.

Object-place recognition (OPR)

For this task, we used a previously established design that has been shown to be hippocampus dependent (Havekes et al., 2012; Oliveira et al., 2010). Mice (n = 31) were handled for 2 minutes each day, for 6 consecutive days leading up to experimentation. The task was conducted in a grey rectangular box (40 cm x 30 cm x 30 cm) built of polyvinyl chloride plastic. Mice were placed in the empty box for 6 minutes for habituation. Mice were then removed and placed back in the home cage. After 3 minutes, mice were placed in the box with 3 different objects (a 100 ml glass bottle, a white cylinder, and a metallic rectangular tower) for 3 consecutive 6-minute training sessions. Each training session was separated by a 3-minute interval during which the animals were returned to the holding cages. At completion of the training sessions, NSD mice were left undisturbed in their home cages and SD mice were deprived of sleep by gentle handling. Twenty-four hours following the training session, mice were re-introduced to the spatial context in a single test session. In this session, one of the objects was repositioned (the displaced object: DO), thereby changing the spatial configuration of the objects in the box. Mice were allowed to explore objects for 6 minutes. Exploration was recorded during training and testing on a digital camera for subsequent scoring of time spent exploring objects. Exploration of the objects was defined as the amount of time mice were oriented toward an object with their noses within 1 cm of it, and was measured by an experimenter who was blinded to the experimental history of the mouse.

The change in preference of object exploration between the last training session and test session was used as an index for spatial memory. Preference for an object during the training and test session was determined by calculating the percentage of time spent exploring a particular object versus total time spent exploring all objects. The formula for this measure is as follows: [TEST (DO/(DO + NDO) x 100]- [3rd TRAINING SESSION (DO/(DO+NDO) x 100], where DO = time spent exploring the displaced object and NDO = time spent exploring the non-displaced object. A positive change in percentage exploration of displaced object between test session and the final training session indicates preference for exploring displaced object. This is indicative of an accurate memory for the original locations of objects and that the mice were able to recognize the object's displacement. No change or a negative change in time spent exploring the displaced object in the test session compared to the last training session indicates inability to recognize the new spatial configuration.

Electrophysiology

To assess the effects of SD on hippocampal LTP, electrophysiological recordings were performed as described previously (Vecsey et al., 2009). Mice (n = 7) were either left undisturbed in their home cages (NSD control group), or subjected to 5 hours of SD. Immediately after this manipulation, SD and time-matched NSD mice were sacrificed by cervical dislocation, and their hippocampi were dissected rapidly in iced oxygenated artificial cerebrospinal fluid (aCSF; 124 mM NaCl, 4.4 mM KCl, 1.3 mM $MgSO_4$*$7H_2O$, 1 NaH_2PO_4*H_2O, 26.2 mM $NaHCO_3$, 2.5 mM$CaCl_2$*$2H_2O$, 10 mM glucose). 400 μm thick transverse hippocampal slices were prepared using a Stoelting tissue chopper (Stoelting Co. Wood Dale, IL) and placed in an interface chamber and continuously perfused with oxygenated aCSF while they equilibrated for at least 1.5 hours at 28.0ºC before starting

electrophysiological recordings. Single pathway recordings were made using a single bipolar stimulating electrode (A-M Systems; 0.002 inches diameter nichrome wire) placed in the stratum radiatum of the CA1 subfield to elicit action potentials in the axons of CA3 pyramidal neurons. An aCSF-filled glass microelectrode (A-M Systems; 1.5 mm x 0.85 mm) with a resistance between 0.5 and 3 MΩ placed in the stratum radiatum region of CA1 was used to record the resulting field excitatory post-synaptic potential (fEPSP). Data were acquired and analyzed using Clampex 8.2 software (Molecular Devices, Palo Alto, CA). Peak fEPSP amplitude was required to be at least 5 mV, and stimulus intensity was set to produce 40% of the maximal response. Test stimulation occurred once every minute. A 20-minute baseline period was recorded in each experiment prior to stimulation. To examine late-phase LTP (L-LTP), 4 trains of stimuli at 100 Hz for 1 second were delivered 5 minutes apart, otherwise known as spaced 4-train high frequency stimulation. Recordings continued for at least 2 hours after LTP induction. Initial fEPSP slopes were normalized against the average of the 20 baseline traces. Input-output characteristics in area CA1 were investigated by recording the fEPSPs elicited by stimuli of increasing intensity after maximum fEPSP was determined. The initial fEPSP slopes were plotted against the amplitudes of corresponding presynaptic fiber volleys and fit with linear regressions. The maximum elicited fEPSP slope was also recorded as a measure of synaptic strength. Paired-pulse facilitation, a short-term form of synaptic plasticity and a measure of presynaptic function, was measured in slices from NSD and SD groups. Paired stimuli were delivered with varying interpulse intervals (300, 200, 100, 50, and 25 ms) and the fEPSP slope from the 2nd stimulus was plotted relative to the slope from the 1st stimulus to give the facilitation ratio.

Biochemistry

For cAMP assays, NSD and 5-hour SD mice were sacrificed and hippocampal tissue was dissected. Samples were stored at -80 ºC until assayed. For the assay itself, a cAMP EIA kit was used (Enzo-Life Sciences). Using this kit, cAMP levels were calculated for each sample and normalized to protein levels.

Statistical Analysis

Two way repeated measures ANOVA was used to analyze behavior in the activity monitoring paradigm. Two-way ANOVA was used to analyze spatial preference in the OPR task, with sleep condition and genotype as factors and delta percent exploration as the dependent variable. Two-way ANOVA was also used to analyze freezing behavior in the CFC task, with sleep condition and genotype as factors and percent freezing as the dependent variable. Student's t-test was used to analyze input output curves. One-way repeated measures ANOVA was used to analyze paired pulse facilitation. Two-way ANOVA was used to analyze cAMP levels, with sleep group and genotype as factors and cAMP measurements as the dependent value. Tukey's multiple comparison was selected for *post hoc* analysis. All statistical analyses were performed using SAS 9.3 software (SAS Institute Inc., Cary, NC). A probability level of $p < 0.05$ was considered significant. Data are presented as mean ± SEM.

Results

Loss of PDE4A disrupts periods of activity during the dark period.

Sleep deprivation dysregulates sleep-wake homeostasis. PDE4A5 has been shown to increase as a result of sleep deprivation. The ability of sleep deprivation to increase PDE4A5 suggests that this enzyme may be a critical regulator of sleep-wake homeostasis. To explore this possibility, we examined what effect loss of PDE4A has on activity across the light/dark cycle. There was an effect of time in percent of activity (**Fig.**

3.1.). Animals displayed increased activity during the dark period in comparison to the light period; $F_{(11,108)} = 299.53$, $p < 0.05$. There was no main effect of genotype on activity across the light-dark cycle; $F_{(1, 108)} = 0.23$, $p = 0.63$. However, there was a significant interaction between genotype and time, $F_{(11, 108)} = 4.93$, $p < 0.05$. Tukey's *post hoc* test revealed that activity levels differed between genotypes at ZT 16 ($t_{(108)} = -4.91$, $p < 0.05$) ZT 22 ($t_{(108)} = 4.03$, $p < 0.05$). The percentage of time PDE4A$^{-/-}$ mice spent inactive during the light period did not differ from WT control animals. However, during the dark period PDE4A$^{-/-}$ mice displayed less exaggerated changes between periods of activity and inactivity in comparison to WT animals, specifically at ZT 16 and ZT 22 (WT: ZT 0 = 85.2 ± 0.96%, ZT 2 = 86.6 ± 1.01%, ZT 4 = 86.1 ± 1.79%, ZT 6 = 89.9 ± 1.75%, ZT 8 = 90.8 ± 0.75%, ZT 10 = 86.2 ± 1.37%, ZT 12 = 46.0 ± 1.84%, ZT 14 = 49.9 ± 1.12%, ZT 16 = 43.9 ± 1.58%, ZT 18 = 63.1 ± 1.85%, ZT 20 = 71.4 ± 2.13%, ZT 22 = 61.5 ± 0.88%; PDE4A$^{-/-}$: ZT 0 = 86.2 ± 1.28%, ZT 2 = 88.7 ± 0.55%, ZT 4 = 86.5 ± 1.25%, ZT 6 = 88.3 ± 1.28%, ZT 8 = 89.2 ± 1.03%, ZT 10 = 84.1 ± 1.34%, ZT 12 = 45.5 ± 1.50%, ZT 14 = 51.3 ± 1.47%, ZT 16 = 52.4 ± 1.08%, ZT 18 = 64.4 ± 1.48%, ZT 20 = 66.6 ± 1.12%, ZT 22 = 54.5 ± 2.00%).

Loss of PDE4A does not rescue sleep deprivation-induced impairments previously seen in contextual fear conditioning.

We wanted to determine if PDE4A is sufficient for sleep deprivation to impair memory in contextual fear conditioning. We chose to use this task because memory impairment due to sleep deprivation was previously observed and rescued by rolipram in this task. Neither sleep deprivation nor genotype appeared to affect long-term memory in this task (**Fig. 3.2.**: WT NSD: 32.9 ± 17.1%, WT SD: 29.6 ± 8.12%, PDE4A$^{-/-}$ NSD: 39.6 ± 28.6%, PDE4A$^{-/-}$ SD: 18.6 ± 9.65%). Although sleep deprivation has previously been shown to

impair memory in this task, we did not observe that effect in this experiment. This was likely due to small sample size in each experimental condition.

PDE4A deficiency rescues spatial memory impaired by sleep deprivation.

Pharmacological inhibition of PDE4 using rolipram rescues fear-associative contextual memory, another form of memory that relies on the hippocampus. We wanted to determine if loss of PDE4A could rescue hippocampus-dependent spatial memories sensitive to sleep deprivation by examining loss of PDE4A on sleep deprived animals performing the OPR task, a measure of spatial memory. Deletion of PDE4A rescued memory in OPR after sleep deprivation (WT NSD: DO = 24.09 ± 5.49%, NDO = -12.04 ± 4.16%; WT SD: DO = 2.11 ± 3.29%, NDO = -0.42 ± 3.54%; PDE4A$^{-/-}$ NSD: DO = 25.69 ± 5.78%, NDO = -12.85 ± 3.75%; PDE4A$^{-/-}$ SD: DO = 14.05 ± 3.44%, NDO = -7.03 ± 2.98%) (**Fig. 3.3.**). There was no main effect of genotype on memory, $F_{(1,54)} = 0.25$, $p = 0.62$. Type of object (DO and NDO) affected exploratory behavior for this particular task, $F_{(1,54)} = 63.45$, $p < 0.05$, indicating preferred exploration of the DO compared to the NDO. A significant interaction existed between genotype and sleep deprivation suggesting that while sleep deprivation affected WT animals, PDE4A$^{-/-}$ mice were resistant to the effects of sleep deprivation on spatial memory, $F_{(4,54)} = 5.24$, $p < 0.05$. Loss of PDE4A function rescues OPR memory, suggesting that sleep deprivation requires PDE4A signaling to impair cognitive performance.

Loss of PDE4A does not affect basal synaptic transmission.

We wanted to examine synaptic plasticity after sleep deprivation in the PDE4A$^{-/-}$ mice. Prior to this experiment, we determined if PDE4A deletion could disrupt neural transmission. Input/output curves did not differ between PDE4A$^{-/-}$ mice and WT mice

(**Fig. 3.4. A**; $t_{(13)}$ = 0.25, p = 0.80). Paired pulse *facilitation* (PPF) was not altered in PDE4A$^{-/-}$ compared to the WT group (**Fig. 3.4. B**; $F_{(4,45)}$ = 0.31, p = 0.87).

Loss of PDE4A does not rescue impairment in synaptic plasticity that accompanies sleep deprivation.

Because rolipram has been shown to rescue sleep deprivation-related disruption in hippocampal synaptic plasticity, we examined LTP after sleep deprivation in PDE4A$^{-/-}$ mice to determine if PDE4A, specifically, was responsible for the rescue of L-LTP. We did not see any drastic changes due to deletion of PDE4A$^{-/-}$ in potentiation of the slice or the effect that sleep deprivation has on the potentiation of the slice compared to slices from WT animals (WT NSD = 164.5 ± 52.5%, WT SD = 126%; PDE4A$^{-/-}$ NSD = 148.5 ± 15.7%, PDE4A$^{-/-}$ SD = 129.0% ± 24.7%). This suggests that this particular type of LTP may not be affected by PDE4A manipulation (**Fig. 3.4. C** and **3.4. D**).

PDE4A deficiency does not affect basal levels of cAMP in the hippocampus.

Prior work has shown that inhibiting PDE activity with use of the xanthine derivative, 3-isobutyl-1-methylxanthine (IBMX), or rolipram results in an increase in cAMP levels in area CA1 of the hippocampus. Because inhibition of PDE leads to an increase of cAMP, loss of PDE4A could potentially increase basal levels of cAMP. We explored this possibility by measuring whole tissue hippocmpal basal levels of cAMP in PDE4$^{-/-}$ mice compared to WT animals, with or without sleep deprivation. cAMP levels did not appear to change as a result of genotype (**Fig. 3.5.**). Unlike what was previously observed in the CA1 region of the hippocampus, sleep deprivation did not appear to alter whole hippocampus levels of cAMP (**Fig. 3.5.**). Loss of PDE4A also did not appear to interfere with the effect of sleep deprivation on cAMP levels in the hippocampus (**Fig. 3.5.**: WT NSD = 20.9 ± 3.33, WT SD = 20.8 ± 2.37, PDE4A$^{-/-}$ NSD = 19.5 ± 2.90, PDE4A^{-}

$^{/-}$ SD = 26.7 ± 4.70). This suggests that sleep deprivation may not be a powerful enough manipulation to disturb basal cAMP levels in tissue collected from either WT or PDE4A$^{-/-}$ mice.

Discussion

Our study investigated whether PDE4A was sufficient to induce the effects of sleep deprivation on memory. We found that loss of PDE4A disrupted activity during the dark period, when rodents are active (**Fig. 3.1.**). We also found that loss of PDE4A function rescues spatial memory deficit caused by sleep deprivation (**Fig. 3.3.**). Although pharmacological inhibition of PDE4 function previously rescued fear memory induced by sleep deprivation, we found no direct evidence that loss of PDE4A could rescue the impairment in fear memory produced by sleep deprivation (**Fig. 3.2.**). We found normal synaptic transmission in PDE4A$^{-/-}$ mice suggesting that presyanptic input was not disrupted by this loss of this particular enzyme (**Fig. 3.4. A** and **3.4. B**). We were unable to assess if loss of PDE4A can rescue the plasticity deficits previously observed in area CA1 of the hippocampus after periods of sleep loss due to our small sample size (**Fig. 3.4. D**). Basal levels of cAMP in whole hippocampal tissue were not perturbed by our sleep deprivation manipulation or by loss of PDE4A (**Fig. 3.5.**). This suggests that the PDE4A gene does not affect the certain aspects related to the consequences of sleep deprivation. Some of these results indicate a role for PDE4A in regulation of the negative consequences of sleep loss on memory.

Sleep-wake behavior is highly complex and controlled by a multitude of genes also involved in learning and memory, including BDNF, CREB, and Homer1A (Graves et al., 2003b; Prospéro-García and Méndez-Díaz, 2004; Naidoo et al., 2012). BDNF and CREB are targets for downstream phosphorylation by cAMP signaling and Homer1A is

regulated by CREB activity (Delghandi et al., 2005; Ji et al., 2005). These genes appear to regulate NREM sleep during the active period. Because cAMP level is regulated by cAMP specific PDE4 enzymatic activity, we wondered if PDE4 activity is involved in the regulation of sleep (Fujimaki et al., 2000; Vecsey et al., 2009). We examined the effect of loss of PDE4A on sleep-wake behavior. We used the activity monitoring paradigm to assess sleep-wake behavior because in the broadest sense, sleep is defined as a period of inactivity. Loss of PDE4A disrupted sleep during the dark period, also known as the active phase for rodents. Because PDE4A potentially mediates genes known to regulate sleep-wake homeostasis, it was unsurprising that PDE4A contributes to sleep-wake regulation (**Fig. 3.1.**). However, it was surprising that the modulation was not in one direction of either increased or decreased sleep during the active period. Future studies are needed to more closely examine the role of this gene in modulation of NREM and REM sleep during the dark period.

Because we observed that PDE4 inhibition did not rescue impaired memory in CFC, we did not predict that loss of PDE4A would not rescue impairments in CFC due to sleep deprivation (**Fig. 3.2.**). In contrast, Vecsey and colleagues observed that the sleep-deprivation induced impairment of memory in CFC was rescued by rolipram inhibition of PDE4 (Vecsey et al., 2009). This discrepancy may be due to our significantly smaller sample size, which may explain why we did not observe an impairment in CFC caused by sleep deprivation in WT animals. The robustness of the task may be a factor as previous use of this task required extensive sample size to observe the effect of sleep deprivation on memory. Instead of recruiting a large sample size to determine the sufficiency of PDE4A for CFC, we decided to examine PDE4A function in sleep deprivation by using the more robust hippocampus-dependent spatial memory task, OPR. The observation that PDE4A$^{-/-}$ mice were resistant to the effects of sleep

deprivation on spatial memory suggests that this particular enzyme regulates the effects of sleep deprivation on hippocampus-dependent spatial long-term memory (**Fig. 3.3.**). Previously, pharmacological inhibition of all classes of PDE4 genes rescued behavior disrupted by sleep deprivation. We thought that PDE4A gene was responsible for the disrupted behavior caused by sleep deprivation because protein expression of the isozyme, PDE4A5, increased as a result of sleep deprivation. In line with these results, loss of PDE4A blunted the effects of sleep deprivation on memory.

Pharmacological inhibition of PDE4 previously rescued reduction in cAMP caused by sleep deprivation. Because we knew that PDE4 was involved in sleep deprivation-regulation of cAMP, we wanted to determine if PDE4A was sufficient to reduce cAMP after sleep deprivation. We found that 5 hours of sleep deprivation did not affect cAMP levels in whole hippocampal samples (**Fig. 3.5.**). Although sleep deprivation previously decreased cAMP levels in mice, the difference was not as dramatic as observed with induction levels of cAMP with forskolin (Vecsey et al., 2009). The small decline in levels of cAMP was also specific to area CA1 of the hippocampus from a slice preparation (Vecsey et al., 2009). Reduction of basal levels of cAMP may have been washed out by examination of whole hippocampus. We also did not observe changes in cAMP in PDE4A$^{-/-}$ mice (**Fig. 3.5.**). This null result may be explained by our experimental design, which examined global levels of cAMP rather than localized changes in cAMP. PDE4 isozymes each have a unique N-terminal localization sequence that directs targeting to a specific set of protein complexes. The localization of these enzymes leads to sequestering of cAMP signaling in specific intracellular domains rather than a general, global degradation of cAMP throughout the cell (Houslay, 2010). This localization of cAMP calls for a more targeted technique to determine if cAMP levels change with loss of PDE4A activity.

Our preliminary examination of synaptic plasticity requires further exploration into the effect of PDE4A on spaced 4-train LTP in slices obtained from previously sleep deprived animals. Although our sample size was low, it appears 5 hours of sleep deprivation reduces LTP (**Fig. 3.4.**), which would replicate prior findings (Kopp et al., 2006; Vecsey et al., 2009). It is not directly apparent from these preliminary recordings whether loss of PDE4A rescues the effect of sleep deprivation. Future investigation is needed to determine the sufficiency of the PDE4A gene. Although pharmacological inhibition of PDE4 with rolipram resulted in rescuing sleep-deprivation induced impairment of LTP (Vecsey et al., 2009), this rescue is not necessarily due to loss of PDE4A.

Surprisingly, loss of PDE4A did not affect memory without sleep deprivation. This is possibly due to compensatory mechanisms resulting from up-regulation of other PDE4 genes, namely PDE4D that is highly expressed in the hippocampus (Pérez-Torres et al., 2000). However, we did not examine the effect of loss of PDE4A on expression of PDE4D and PDE4B genes, which are also expressed in the hippocampus. Future research should examine the contribution of these other PDE4 genes in PDE4A$^{-/-}$ mice to determine what role they play in preserving memory in these mice. Compensatory mechanisms could reasonably block knockout effects on memory. However, the impact of sleep deprivation may rely solely on PDE4A signaling. In this case, compensatory mechanisms may not effectively account for the loss of PDE4A to allow sleep deprivation to exert effects on hippocampus dependent memory. The contribution of these other PDE4 genes should be further investigated in the hippocampus in respect to sleep deprivation.

Prior to this work, we knew that PDE4 mediated the effects of sleep deprivation on memory. However, we remained uncertain about which PDE4 gene was responsible

for these negative consequences on hippocampus-dependent memory. We determined in this study that PDE4A is a critical gene for memory impairments induced by sleep deprivation. Modulation of some aspects of sleep deprivation on hippocampus-dependent memory by PDE4A requires more in depth study. Although we found that PDE4A mediates some of the effects of sleep deprivation, the question remains whether PDE4A5 isoform is driving the effects of sleep deprivation on hippocampal function. Future endeavors to examine the role of PDE4A5 should involve development and use of a more targeted deletion or knockdown of this enzyme in the hippocampus. A dominant negative approach has been developed to determine the functional role of particular PDE4 isozymes (Baillie et al., 2003; McCahill et al., 2005). Implementing the dominant negative approach to reduce catalytic activity could be one potential avenue to determine whether the effects of sleep deprivation are modulated by PDE4A5 expression. This approach would enable specific investigation of the PDE4A5 isoform, which is the isoform originally implicated in the effects of sleep deprivation on hippocampal function. From the studies described in this chapter, we now know that PDE4A mediates the effects of sleep deprivation in memory, and regulates sleep-wake homeostasis.

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Author Contributions

Experiments were conceived and designed by Toni Prince and Dr. Ted Abel. Electrophysiological and behavioral experiments were carried out by Toni-Moi Prince This chapter was written by Toni-Moi Prince with comments and editing by Christopher Angelakos, Dr. Jennifer Choi and Dr. Ted Abel.

FIGURE 3.1.

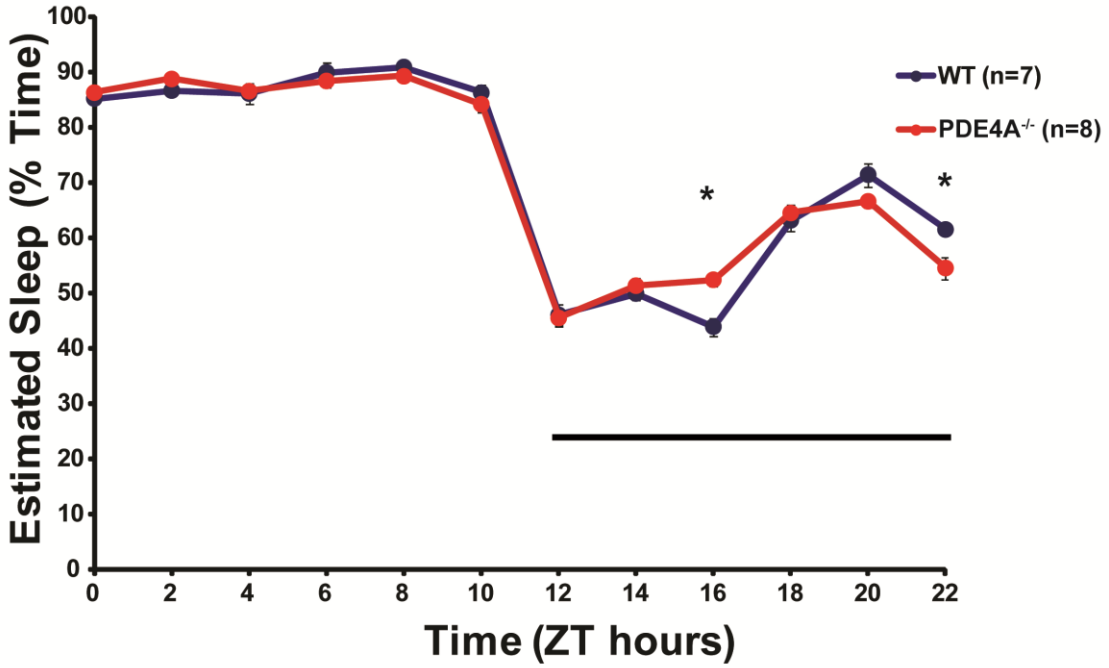

Figure 3.1. *PDE4A deletion modulates activity during the dark the dark period*.

Percent time spent asleep shown in 12 x 2 hour bins across light/dark cycle. Solid line represents the dark period/active phase for rodents. Amount of sleep achieved differed by time of day ($F_{(1,108)}$ = , $p < 0.05$). Time spent asleep did not differ between WT (n = 7) and PDE4A$^{-/-}$ mice (n = 8) ($F_{(1,108)} = 0.23$, $p = 0.63$). PDE4A$^{-/-}$ mice displayed differences in time asleep during the active period compared to WT mice ($F_{(1,108)} = 4.93$, $p < 0.05$). Error bars indicate ± SEM.

FIGURE 3.2.

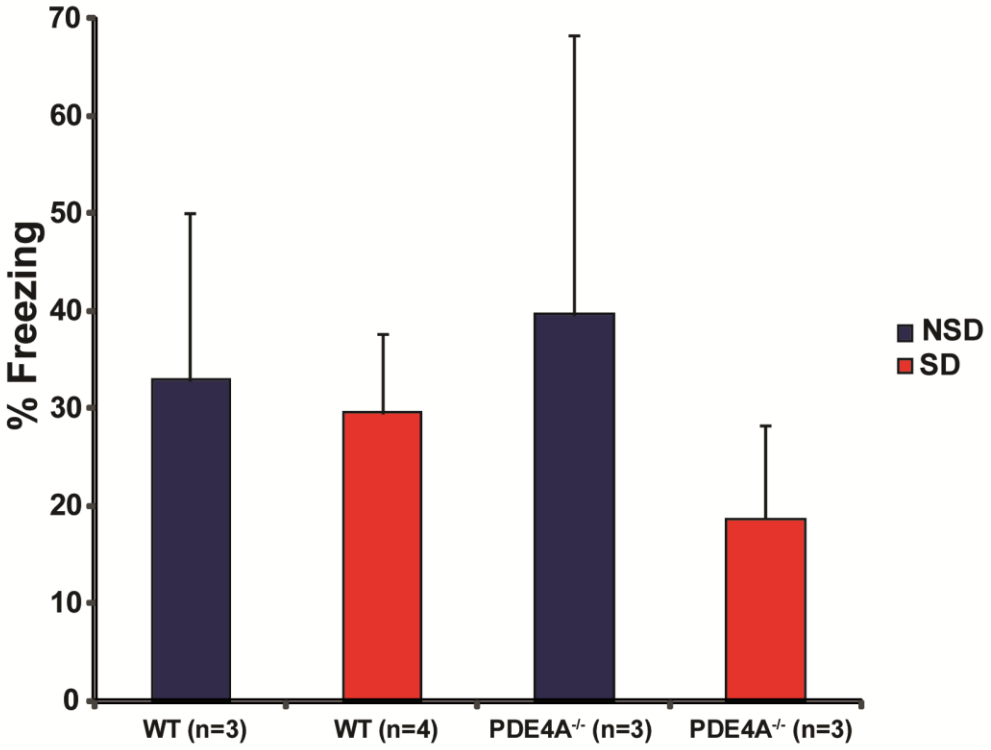

Figure 3.2. *5 h sleep deprivation does not impair contextual fear conditioning in PDE4A$^{-/-}$ mice.*

The effect of genotype on sleep deprivation did not affect mean percent freezing. Mean percent freezing did not differ between WT (n = 7) and PDE4A$^{-/-}$ group (n = 6). Sleep deprivation did not affect mean percent freezing. SD animals (n = 7) froze to a similar extent as NSD animals (n = 6). Error bars indicate ± SEM.

FIGURE 3.3.

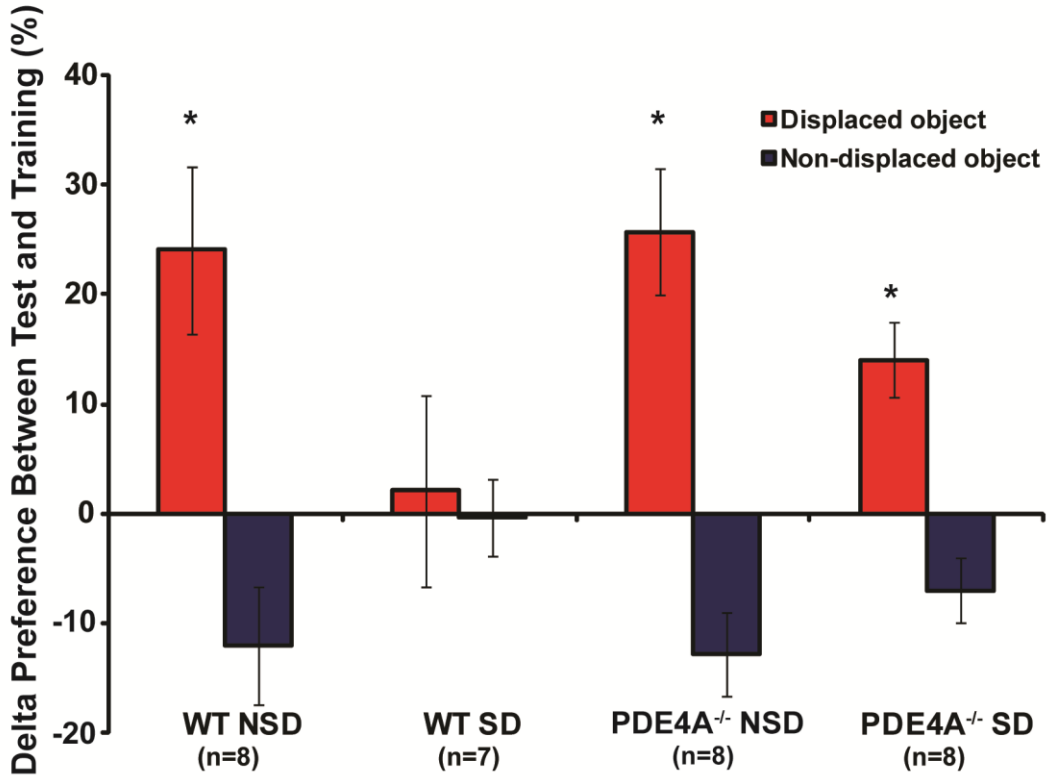

Figure 3.3. *Deletion of PDE4A rescues object-place recognition memory after sleep deprivation.*

Mean delta percentage exploration of the DO increased compared to exploration of the NDO for NSD animals in both the WT group (n = 8: $F_{(4,54)} = 5.24$, $p < 0.05$; $t_{(54)} = 5.96$, $p < 0.05$) and PDE4A$^{-/-}$ group (n = 8: $F_{(4,54)} = 5.24$, $p < 0.05$; $t_{(54)} = 6.36$, $p < 0.05$). Mean delta percentage exploration of the DO was not significantly different than NDO for the SD animals in the WT (n = 7: $F_{(4,54)} = 5.24$, $p < 0.05$; $t_{(54)} = 1.46$, $p = 0.83$). Mean delta percentage exploration of the DO was significantly different than NDO for SD animals in PDE4A$^{-/-}$ group (n = 8: $F_{(4,54)} = 5.24$, $p < 0.05$; $t_{(54)} = 3.48$, $p < 0.05$). Error bars indicate ± SEM.

FIGURE 3.4.

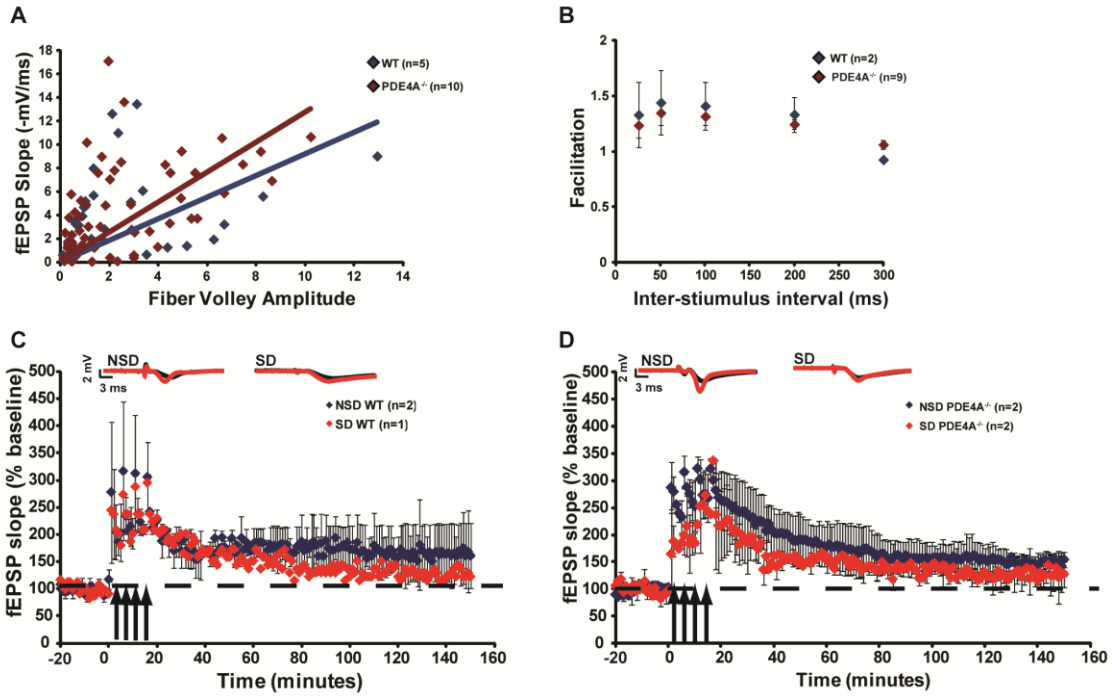

Figure 3.4. *Deletion of PDE4A does not affect synaptic transmission or synaptic plasticity.*

(A) Input-output curve relating the amplitude of the presynaptic fiber volley to the initial slope of the corresponding fEPSP at various stimulus intensities was not altered in slices from WT (n = 5) compared to slices from PDE4A$^{-/-}$ (n = 10) ($t_{(13)}$ = 0.25, p = 0.80). *(B)* Paired pulse facilitation, a short-term form of synaptic plasticity, did not differ in slices obtained from either WT group or PDE4A$^{-/-}$ group ($F_{(4,45)}$ = 0.31, p = 0.87). *(C)* There was a trend toward reduced L-LTP as a result of sleep deprivation in WT mice (NSD = 2, SD = 1). *(D)* L-LTP was not affected by sleep deprivation in PDE4A$^{-/-}$ mice (NSD = 2, SD = 2). Error bars indicate ± SEM.

FIGURE 3.5.

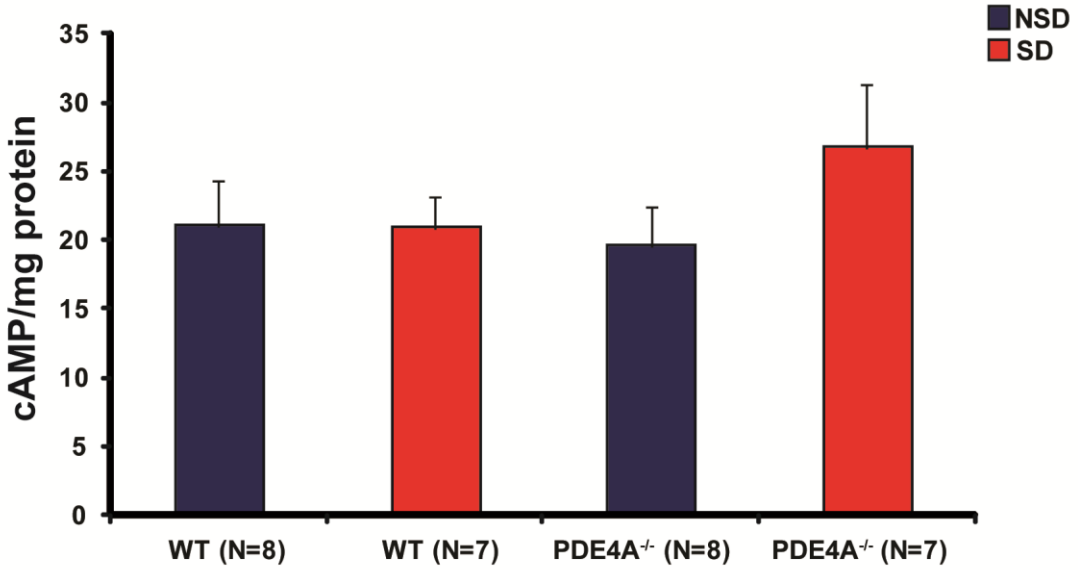

Figure 3.5. *5 h sleep deprivation does not affect cAMP levels in whole hippocampus tissue.*

Mean levels of cAMP was not affected by the effect of genotype on sleep deprivation. Mean levels of cAMP did not differ between the WT group (n = 15) and PDE4A$^{-/-}$ group (n = 15). Mean levels of cAMP did not differ between SD and NSD. Error bars indicate ± SEM.

CHAPTER FOUR

GENERAL DISCUSSION AND FUTURE DIRECTIONS

Sleep loss results in many devastating consequences including impairments in cognitive processes such as attention and memory formation. It is unlikely, however, that individuals will alter their schedule to attain sufficient quality sleep, so it is necessary to develop treatments and therapeutics to combat these cognitive deficits. To develop new treatment options, it is imperative to understand the underlying mechanisms by which sleep deprivation or sleep can impair or enhance memory, respectively. In this dissertation, we were particularly interested in understanding the effects of sleep deprivation on hippocampal functioning. To understand how sleep deprivation impairs memory, we delved into two avenues of study. First, we used a timecourse experiment to explore the time window where sleep deprivation is disruptive to memory consolidation, which can be considered the kinetics of sleep deprivation. We also investigated novel molecular targets that enable sleep deprivation to be effective at disrupting memory, which can be considered the mechanics of sleep deprivation.

4.1. Kinetics: Temporal dynamics of sleep deprivation that impairs memory consolidation

In **Chapter 2**, we found that timing of sleep deprivation is important in impairing hippocampus-dependent long-term memory consolidation. Previously, immediate post-training sleep deprivation of 4 hours or greater had been shown to impair hippocampus-dependent long-term memory (Smith and Rose, 1997). However, later periods of sleep deprivation administered hours after training had no effect on memory consolidation (Smith and Rose, 1997; Graves et al., 2003a; Bjorness et al., 2005; Palchykova et al., 2006; Hagewoud et al., 2010c). These earlier findings suggests that memory is sensitive to immediate sleep deprivation post-training, but resistant to later periods of sleep deprivation. Although these results support the existence of a discrete time window

where long-term memory is sensitive to sleep deprivation, prior work did not demonstrate whether a shorter time-window of sleep deprivation could still impair long-term memory. To determine the most effective period for sleep deprivation to disrupt long-term memory within the 4-hour time frame from, we tested varied lengths of sleep deprivation within this window. We found that a delayed 3-hour period of sleep deprivation from 1 to 4 hours (ZT 2-5) after training disrupts hippocampus-dependent spatial memory and synaptic plasticity. In contrast, an immediate 3-hour period of sleep deprivation from 0 to 3 hours after training (ZT 1-4) had no effect on either memory or synaptic plasticity. These findings demonstrate that a specific 3-hour acute period of sleep deprivation during consolidation is capable of disrupting processes required for hippocampal function. These results suggest that both the *duration* and the *timing* of sleep deprivation are critical factors for memory disruption.

Highlighted in these findings is the importance of timing of sleep deprivation and possibly sleep. Timing of sleep has now come to the forefront of research in the sleep field. Previously, research focused almost exclusively on duration of sleep, showing that memory benefited from longer durations of sleep (Stickgold et al., 2000; Walker et al., 2003). The general assumption was that sleep needed to occur immediately after acquisition to stabilize memory because delayed sleep deprivation was ineffective at impairing memory. However, more recent studies have observed that a short delay between learning and sleep has the potential to optimize memory consolidation. The benefit of delayed sleep has been shown in humans performing a declarative task. If sleep occurred 3 hours after learning, it was effective at stabilizing memory (Gais et al., 2006; Talamini et al., 2008). This demonstrates that sleep may not need to occur immediately after training in order to support memory formation (Walker et al., 2003; Korman et al., 2007). The study described in **Chapter 2** is the first to characterize the

critical time window where sleep deprivation during consolidation impairs both synaptic plasticity and memory in rodents, providing evidence for the importance of timing in sleep deprivation.

Despite the copious evidence for the importance of sleep for memory, questions remain as to why sleep is so important for spatial memory during this specific 3-hour period. Several potential avenues exists that may explain our findings, including possible differences in sleep architecture among time windows, effects of training on sleep, or contributions to hippocampal function. To examine the role of sleep architecture, we analyzed the components of sleep in mice during ZT 1 to ZT 5 without prior training. Sleep deprivation from ZT 2-5 may have been more effective at impairing memory if animals sleep more during this period than ZT 1-4. However, we observed no differences in REM and NREM during these hours, suggesting that mice being sleep deprived from ZT 2-5 are not losing more sleep than animals sleep deprived from ZT 1-4. Although these findings suggest no difference in sleep across this time period, prior training may change sleep architecture. One possibility that requires exploration in the future is whether sleep architecture during ZT 2-5 differs from ZT 1-4 after behavioral training.

Memory benefits from rapid eye movement (REM) sleep as well as non-rapid eye movement (NREM) sleep, specifically slow wave sleep (SWS) (Barrett and Ekstrand, 1972; Smith, 1995; Plihal and Born, 1997, 1999). Support for the beneficial role of these sleep stages on memory comes from observations that reactivation of hippocampal neurons previously active during acquisition occurs in both REM and SWS after acquisition(Pavlides and Winson, 1989; Wilson and McNaughton, 1994; Poe et al., 2000; Louie and Wilson, 2001; Ji and Wilson, 2007). REM sleep is characterized by multiple patterns of neuronal field potentials, including ponto-geniculo-occipital (PGO) waves and

theta frequency oscillations, are characteristic of REM sleep. Previous research suggests that both of these REM sleep field potentials support the consolidation process (Datta, 2000; Buzsáki, 2002; Ulloor and Datta, 2005; Datta et al., 2008). Field potentials that occur during SWS have also been found to be beneficial for memory consolidation. SWS is filled with cortical slow oscillations, thalamo-cortical spindles, and hippocampal sharp-wave ripples. These 3 types of field potentials are linked to stabilization of memory (Clemens et al., 2005, 2006; Marshall et al., 2006; Axmacher et al., 2008). Interestingly, a temporal relationship seems to exist between the SWS field potentials that enhance consolidation. For instance, ripples and spindles increase during the up state of slow oscillations and decrease with down state of slow oscillations. This is hypothesized to allow information to flow between the hippocampus and the neo-cortex, an area known to aid memory stabilization (Steriade, 2006). We did not measure hippocampal ripples or cortical spindle activity during post-training sleep, but loss of SWS activity during consolidation could be a potential mechanism underlying the effects of sleep deprivation on memory. Sleep loss during the delayed 3-hour period possibly contributes to loss in coupling of SWS field potentials in comparison to immediate sleep deprivation. This loss of coupling potentially impairs memory. Although we found that sleep did not differ between these two periods, prior training could affect the amount, type of sleep, and temporal relationship of field potentials during these two time periods. It has been reported that sleep changes as a result of prior training. However, conflicting results exists as to the nature and direction of these changes in sleep due to prior training events (Tang et al., 2004, 2005a, 2005b, 2007; Hellman and Abel, 2007). Based on these previous findings, post-training sleep architecture during these 2 periods is an area worth examining in the future.

Assessing sleep architecture after post-training sleep deprivation could also enlighten us to why we observed memory impairments based on timing of sleep deprivation. We observed that more manipulation was required to maintain wakefulness in the animals in the first hour of the delayed sleep deprivation group compared to the immediate sleep deprivation group, suggesting that this later sleep deprivation had accrued high sleep pressure. When animals are allowed to sleep after a period of sleep deprivation, animals exhibit increased sleep pressure and accompanying periods of highly consolidated sleep bouts, referred to as 'rebound sleep', which can indicate homeostatic imbalance (Nelson et al., 2013). By examining sleep post sleep deprivation, we can compare rebound sleep after immediate 3-hour sleep deprivation to rebound sleep after delayed 3-hour sleep deprivation. This experiment could potentially provide insight into why hippocampal function is more sensitive to the delayed 3-hour sleep deprivation. Increased intensity of delta waves during rebound sleep post delayed sleep deprivation would indicate that this period of sleep deprivation is a more powerful manipulation than the immediate sleep deprivation, as well as explain why the same duration of sleep deprivation could cause deficits in one time window but not the other.

Most rodent studies examine the effects of sleep deprivation on memory during the rest period, when sleep pressure is maximally elevated. However, learning usually occurs during the active period for humans. Exploring how sleep deprivation affects memory during the active period would contribute nicely to our understanding of the relationship between sleep and memory. Newer studies have begun to examine these time of day differences in the effectiveness of sleep. For example, rodent studies have observed that memory particularly benefits from sleep when task learning occurs in the early morning period, during a period of elevated sleep pressure (Binder et al., 2012). A prior study has observed that active-phase consolidation could be disrupted by an

immediate 12 hours of sleep deprivation, but not by 6 hours of sleep deprivation (Hagewoud et al., 2010c). This indicates that a longer duration of sleep deprivation is required to impair memory during the active period. A longer period of sleep deprivation may be necessary to impair memory due to mice sleeping less during the active period. Therefore, a longer duration of sleep deprivation may be necessary to cause the same amount of sleep loss that was shown to impair memory during the rest period. Knowing that a longer period of sleep deprivation is required to impair memory suggests that time windows may be different during the active period in comparison the rest period. Exploration of time windows required to disrupt hippocampal function during the active period could provide more insight into differences in sleep loss at varying times of day.

While our interesting findings underscore the importance of sleep deprivation from 1 to 4 hours after training to disrupt hippocampus dependent memory and synaptic plasticity, we recognize that this finding may be specific to OPR and gentle handling. This time window should not necessarily be expected in all long-term memory tasks or even, specifically, hippocampus dependent tasks. Future work should explore if the same time window of sleep deprivation can successfully impair memory in other hippocampus dependent tasks. Generation of memory impairments by sleep deprivation appears to rely on disruption of critical signaling periods for memory consolidation. However, the time-course of molecular signaling during consolidation varies between tasks, as well as within a task where differing paradigms are employed (Bernabeu et al., 1997a; Bourtchouladze et al., 1998). The differences in the timing of critical signaling required for tasks suggests that time windows for sleep deprivation to disrupt these signaling cascades may also be different from task to task. Unfortunately, the OPR task is one of the few rodent tasks in which hippocampal signaling activity has not been explored during consolidation. Understanding signaling dynamics after OPR training may

answer our question of why these 2 sleep deprivation periods result in different outcomes. Graduate students, Shane Poplawski and Hannah Schoch, from the Abel lab have begun to assess the time course of gene expression involved in memory consolidation after OPR training.

The hippocampus was the focus of this dissertation. However, this brain region is not the only area sensitive to sleep and sleep loss. Functions that rely on prefrontal cortex (PFC) also benefit from sleep (Walker and Stickgold, 2006). The PFC regulates executive functioning, which includes attention, working memory, temporal memory, and behavioral inhibition (Fuster, 1997; Goldberg, 2001). Additionally, the PFC has been shown to interact with the hippocampus in stabilization of long-term memory (Cohen, 2011). Cross-talk between the cortex and the hippocampus is the foundation of active systems consolidation theory. Sleep deprivation disrupts PFC dependent learning (Durmer and Dinges, 2005; Steriade, 2006). Sleep deprivation also enhances excitability and plasticity of PFC neurons (Romcy-Pereira and Pavlides, 2004; Winters et al., 2011; Yan et al., 2011). In the future, we would be interested in determining whether a time window exists in which the PFC is sensitive to sleep loss.

We show that timing matters for total sleep deprivation by gentle handling to disrupt hippocampal function. However, it would be of interest to examine whether the same time window exists for other sleep deprivation techniques to disrupt memory. For example, would we observe the same sensitive time window with REM-specific sleep deprivation? It has already been shown that an immediate 4 hours of REM sleep deprivation can impair long-term memory in a hippocampus-dependent task (Smith and Rose, 1997). It is unknown whether a shorter period of REM sleep deprivation would impair long-term memory. A similar question could be posed in regards to fragmentation of sleep. Recently, the sleep research field has experienced the emergence of an

optogenetic approach to examine the effects of sleep fragmentation on memory (Rolls et al., 2011). Rolls and colleagues demonstrated that an immediate period of sleep fragmentation, using optogenetics to stimulate arousal by activating hypocretin cells, impaired long-term memory in novel object recognition. It would be interesting to investigate whether other types of selective sleep disruption techniques demonstrate similar time windows in which hippocampal function can be impaired.

Sleep deprivation is detrimental to the consolidation stage of memory (Prince and Abel, 2013). Yet, most studies have exclusively focused on just the effects of sleep deprivation on molecular signaling and synaptic plasticity in the hippocampus without examining its impact post-training. To demonstrate this point, we are the first group to demonstrate the impact of post-training sleep deprivation on hippocampal synaptic plasticity. This is surprising because most of the literature compares effects of sleep deprivation on long-term LTP to effects of sleep deprivation on both memory acquisition and consolidation. More studies should use this design to more accurately explore the effects of sleep deprivation on signaling and transcription during the consolidation phase. In order to understand how sleep deprivation impacts consolidation, it is essential to examine molecular signaling pathways. In this vein, we are determined to discover how immediate 3-hour sleep deprivation differs from delayed 3-hour sleep deprivation during consolidation by examining expression of previously identified molecular targets as well as potential molecular targets after these periods of sleep deprivation after OPR training, such as the phosphodiesterase PDE4A5 and the neurotrophin receptor $p75^{NTR}$ (characterized in **Chapter 3** and the **Appendix**) and transcriptional regulation. Dr. Jennifer Choi, has started examining the time course for sleep deprivation-induced changes in transcription and translation. For instance, expression PDE4A5, which was first characterized in the Abel lab as a signaling molecule with activity that increases with

sleep deprivation, has yet to be examined in the context of sleep loss during consolidation. It may be that our delayed period of sleep deprivation from 1h to 4h (ZT 2-5) after training significantly increases expression of PDE4A5, while expression remains unaffected by animals subjected to immediate sleep deprivation and no sleep deprivation.

4.2. Mechanics: Uncharacterized molecular targets that enable sleep deprivation to be effective at disrupting memory

Prior work from the Abel laboratory pointed to one possible signaling complex as a potential culprit for impaired hippocampal function after sleep deprivation. This complex includes PDE4A5, an isoform of the PDE4A gene. In **Chapter 3,** we test the sufficiency of the PDE4A gene during sleep deprivation to impair hippocampus-dependent related functions, and during normal sleep to mediate sleep-wake activity. To examine this, we used a constitutive knockout mouse line, which lacks all isoforms of the PDE4A gene in all tissues. We found that although loss of this gene rescued spatial memory from the effects of sleep deprivation, this rescue was not apparent in hippocampus-dependent fear memory. In contrast to previous reports, sleep deprivation did not impair memory for contextual fear conditioning (CFC) in our hands (Graves et al., 2003a; Vecsey et al., 2009; Hagewoud et al., 2010c). No effect may have been observed because this task requires a larger sample size to see the effect of a brief period of sleep deprivation. We decided to examine the effects of sleep deprivation on the more robust OPR task. However, it would be interesting to determine if loss of PDE4A would have a general effect on memory in this task because loss of its family member, PDE4D, impairs fear-conditioning memory (Rutten et al., 2008).

The impact of loss of PDE4A function on sleep and memory is relatively unexplored territory in comparison to other PDE4 family members. We were the first to explore the role of PDE4A in hippocampal function. PDE4B and PDE4D have been well characterized for their effects related to anxiety, schizophrenia, and depression. Other PDE4 genes have been observed to enhance cognitive function. For instance, loss of PDE4D, which is predominantly expressed in CA1 region of the hippocampus (Pérez-Torres et al., 2000), results in enhanced memory and increased neurogenesis through increased cAMP signaling (Li et al., 2011a), while over-expression of PDE4D impairs memory (Giorgi et al., 2004). Because other PDE4 gene knockouts exhibit altered memory and abnormal growth and development, we were surprised that loss of PDE4A did not result in alterations in memory or behavior (Jin, 1999). The mild phenotype of PDE4A deficient mice could be due to differences in the distribution of the PDE4A compared to PDE4D in the hippocampus, as well as the kinetics of when these enzymes become active. Results from previous findings support this tempered response from PDE4A. For instance, if PDE4D deficient mice are given rolipram no further increase in hippocampus-dependent long-term memory occurs (Li et al., 2011b). No further improvement in memory when rolipram is given to PDE4D deficient mice indicates that PDE4A and PDE4B may not contribute to enhancements in baseline memory. This suggests that PDE4A may not mediate memory until an external factor, such as sleep deprivation, is involved. The difference in the regulation of enzymatic activity between PDE4A and PDE4D likely contributes to amount of cAMP degradation. Loss of PDE4D resulted in a large increase in cAMP in mouse embryonic fibroblasts, while loss of PDE4A resulted in only a minimal increase in cAMP (Bruss et al., 2008). This could explain why we did not observe a difference in cAMP levels from whole hippocampal tissue in PDE4A$^{-/-}$ mice compared to WT.

Neurons express multiple functionally distinct isoforms of PDE4A. Due to splicing and alternative start sites, PDE4A is composed of long, short, super-short, and dead-short isoforms. These isoforms differ in the N-terminal region, which is under the control of specific promoter that affects compartmentalization in the cell (Conti et al., 2007; Houslay, 2010). The long-forms, which are sequestered by anchoring proteins, are activated by PKA mediated phosphorylation. Activation of this enzyme by PKA facilitates degradation of cAMP. Because we did not examine particular isoforms of PDE4A, it is not clear in our study which isoform is responsible for the rescue of spatial memory. One of the long forms of PDE4A, PDE4A5, has been characterized as an enzyme involved in impairing memory during sleep deprivation. However, many of its downstream components remain unidentified. Prior work has shown that PDE4A5 enzyme is recruited by p75 neurotrophin receptor ($p75^{NTR}$) to reduce cAMP-PKA signaling in sciatic nerve tissue (Sachs et al., 2007), suggesting that this may be the case in other tissue such as the hippocampus. $p75^{NTR}$ is phosphorylated at Serine 304 by PKA to translocate the receptor to lipid rafts (Higuchi et al., 2003a). This translocation allows $p75^{NTR}$ to initiate intracellular signaling transduction (Higuchi et al., 2003a). This receptor is in a negative feedback loop with PKA function. When cAMP is elevated, PKA activates $p75^{NTR}$, similar to PDE4A5- activation by PKA (Dodge et al., 2001). The interaction between these PDE4A5 and $p75^{NTR}$ suggests that $p75^{NTR}$ may be a key player in PDE4A5-mediated reduction of cAMP during sleep deprivation. If this is the case, hippocampal function of animals deficient in $p75^{NTR}$ would not be sensitive to sleep deprivation because of blocked interaction between PDE4A5 and this receptor.

In the **Appendix**, we explore the neurotrophin receptor p75 ($p75^{NTR}$), which interacts with PDE4A5 to reduce cAMP levels (Sachs and Akassoglou, 2007). To determine the role of p75NTR in sleep deprivation and memory, we used a constitutive

knockout mouse model to examine whether $p75^{NTR}$ is required for hippocampal memory. The benefit to using this model is that it enables determination of the gene function throughout development of the hippocampus. However, loss of the gene is not only limited to the hippocampus, but to all regions of the body. Because $p75^{NTR}$ is involved in many functions within the body, constitutive loss of the receptor produces global deficits, such as peripheral de-innervation (Lee et al., 1992; Peterson et al., 1999; von Schack et al., 2001). This makes it difficult to interpret the behavior of these animals in many of the behavioral tasks we employ in our lab, and thus, precludes us from concluding anything from the experiments conducted in the study described in the **Appendix**. However, we discuss the possible contributions of both of these targets in terms of potentially mediating sleep deprivation-induced deficits of hippocampal function.

We observed a deficit in CFC in $p75^{NTR-/-}$ mice, but this deficit may be due to innervation deficits as this task requires the animals to sense the shock stimuli with their paws. This is likely to be the case, because we observe a trend towards enhanced memory in the OPR task which is less reliant on peripheral sensory innervation. The contrast in performance in these tasks suggests that $p75^{NTR}$ is needed by other regions outside the hippocampus. While loss of the receptor may have an effect on hippocampus-dependent memory, impaired functioning in other systems confounds our interpretation of these tasks. This demonstrates the need for a more targeted approach to explore the role of $p75^{NTR}$ in memory and sleep deprivation. Recently a conditional knockout has been developed, which could be used to examine $P75^{NTR}$ in the hippocampus (Bogenmann et al., 2011). An even more targeted approach would be a viral knockdown of $p75^{NTR}$ which would allow exploration of the functional role of $p75^{NTR}$ in sub-regions of the hippocampus (Higuchi et al., 2003b). The same issue concerning approach applies to examination of PDE4A. Although we determined that PDE4A is

sufficient for spatial memory and involved in sleep-wake patterns, use of a constitutive knockout mouse does not allow exploration of specific isoforms, such as PDE4A5, that have previously been linked to disruption of memory by sleep deprivation. We think a viral knockdown or an over-expression approach may be more appropriate for the types of questions we are asking. A more targeted approach is required in this instance due to the nature of activation of this enzyme, in which the enzyme is not ubiquitously activated in regulation of cAMP, but rather activation is spatially localized at particular signaling complex (Houslay, 2010; Houslay and Adams, 2010). This suggests that PDE4A isoforms are unlikely to increase globally with sleep deprivation, but instead may have a more fine tuned elevation of enzymatic activation based on region and cell type. Members of the Abel laboratory, spearheaded by Dr. Robbert Havekes, have now begun to examine the role of PDE4A5 in sleep deprivation using a dominant-negative mutant form of PDE4A5 in which the catalytic region is inactive (Baillie et al., 2003; Houslay, 2010) to inhibit cAMP degradation. Overall, viral approaches supersede knockout models in both of these studies because they provide temporal and spatial restrictions. Because this technique is both temporally and spatially restrictive, it avoids the pitfalls of constitutive knockout models, which can be compromised by developmental and somatic phenotypes. Loss of a gene during development often results in either compensatory mechanisms or developmental deficits. Loss of a gene somatically often results in deficits in outside regions that may interfere with the area of interest.

Using the more targeted viral approach in the future will enable investigation into important questions that pertain to both PDE4A5 and $p75^{NTR}$ function. We would like to examine effects of sleep deprivation on both memory and synaptic plasticity in a model that allows both spatial and temporal regulation of $p75^{NTR}$ loss. This approach would allow us to examine whether $p75^{NTR}$ plays a role in hippocampus-dependent memory

impairments caused by sleep deprivation, an experiment we were unable to do with our current model. Interestingly, In **Chapter 3**, we found that PDE4A regulates sleep-wake activity. To continue on this avenue, more-in depth examination of NREM and REM sleep as well as monitoring of behavioral activity without entrainment by light. This would provide valuable information for both the homeostatic and circadian contribution of PDE4A. We would also like to examine which isoform is responsible for these changes in sleep-wake patterns. We are also aware that $p75^{NTR}$ is a clock gene. To determine how the functions of these proteins overlap, we need to examine whether sleep-wake patterns in $p75^{NTR-/-}$ mice match what we observe in $PDE4A^{-/-}$ mice.

The hypothesis that $p75^{NTR}$ and PDE4A activation underlie the memory and plasticity deficits observed with sleep deprivation was explored in **Chapter 3** and the **Appendix,** but critical questions still remain. Future studies should focus on determining whether PDE4A5 localization by $p75^{NTR}$ is required to impair memory after sleep deprivation. It has already been determined that these two proteins work together to reduce cAMP in sciatic tissue (Sachs et al., 2007). However, whether this interaction is necessary for degradation of cAMP in the hippocampus with sleep deprivation is unknown. Neither disruption in PDE4A5 or $p75^{NTR}$ directly address the nature of their functional roles during sleep deprivation. To be able to answer this question, we would want to determine whether sleep deprivation still impairs memory when these proteins are unable to interact. If impairments due to sleep deprivation still exist, this would suggest that sleep deprivation does not require interaction of these proteins to impair memory. This result would be an indication that these proteins exist in different signaling cascades that may be perturbed distinctly by sleep deprivation. Another potential experiment would be to determine whether over-expression of PDE4A5 impairs hippocampal function. This manipulation should essentially be a phenocopy of the

effects of sleep deprivation if PDE4A5 activity underlies the effects of sleep loss on memory. If this is the case, then inhibiting binding of PDE4A5 and $p75^{NTR}$ should restore function. Once again, this last experiment would reveal whether binding of these proteins worked in combination to impair hippocampal function.

Although the field has made many breakthroughs in terms of uncovering hippocampal signaling pathways involved in the effects of sleep deprivation, much has yet to be explored. PDE4A5 has been identified, but little is known about other proteins in this pathway. We began by examining $p75^{NTR}$, but that is not the only target that PDE4A5 interacts with directly or indirectly. Discovering other interactions downstream or upstream of PDE4A5 would enable characterization of potential future targets. Additionally, sleep deprivation may disrupt pathways that seemingly do not overlap. However, cross-over may exist between these signaling pathways disrupted by sleep deprivation. For example, inhibitors of PDE4 serve as general antagonists for adenosine receptors (Ukena et al., 1993). One potential target that warrants future study is the immunophilin XAP-2 (Bolger et al., 2003), which works by binding to the upstream conserved region 2 (UCR2 domain) of PDE4A5. This interaction inhibits PDE4A5 ability to degrade cAMP by attenuating its ability to phosphorylated by PKA. Another target that potentially affects PDE4A signaling and requires future examination in the mechanics of sleep deprivation is AKAP150, an anchoring protein that recruits proteins into a signaling complex (Nijholt et al., 2008; Logue and Scott, 2010). This protein is down-regulated as a result of 6 hours of sleep deprivation (unpublished observation by Dr. Robbert Havekes). In general, identification of other targets will be critical to our understanding of how sleep deprivation disrupts cellular and molecular processes. Dr. Robbert Havekes has begun assessing how sleep deprivation mediates synaptic changes by examining proteins that involved in structural changes in synaptic connections.

As we alluded to in the kinetics section of the discussion, time-course analysis of the effects of sleep deprivation can elucidate how disruption of signaling at particular time periods impairs hippocampal function. Although we did not explore the effects of sleep deprivation during the consolidation stage in the biochemical and synaptic plasticity experiments described in **Chapter 3** and the **Appendix,** we were essentially examining the effects of sleep deprivation on the acquisition stage, which is also sensitive to sleep loss. Therefore examining molecular signaling over the time-course of sleep deprivation before and after training would provide useful information pertaining to how sleep deprivation affects both the acquisition and the consolidation stages of memory. In **Chapter 3** and the **Appendix**, we examined the effects of 5 hours of sleep deprivation on plasticity and cAMP. Although this provides information on what signaling is perturbed by sleep deprivation, we only gain perspective from one time point. It would be valuable to see how cAMP reacts over the course of sleep deprivation as well as PDE4A and $p75^{NTR}$. Not only would it be valuable to understand the kinetics of PDE4A5 and $p75^{NTR}$ separately, but determining the kinetics of the interaction of PDE4A5 and $p75^{NTR}$ during sleep deprivation post-training would also be informative. A time-course of analysis would provide us with more detailed measurement of how signaling changes in the hippocampus over a period of sleep deprivation. A clear understanding of the kinetics will specify a time period of when therapeutics should be active to combat the negative cognitive effects of sleep deprivation.

Concluding Remarks

My focus over the course of this dissertation was to ascertain the fundamental kinetics and mechanics of sleep deprivation. By kinetics, I refer to the minimal critical time window of sleep deprivation that can disrupt hippocampal function. Mechanics is

defined as critical signaling components involved in sleep deprivation disruption of hippocampal function. From these studies, we have determined the minimum time window in which sleep loss during consolidation can impair spatial long-term memory and synaptic plasticity, addressing kinetics. Further, we characterized the role of particular genes thought to be involved in the impact of sleep deprivation on hippocampal function, addressing mechanics. Although these findings are insightful and useful, the impact of sleep deprivation in humans is experienced on a much larger scale than what is examined here. For instance, most individuals suffer from brief periods of sleep loss over multiple days, weeks, and even years instead of the single exposure we explore. Although, repetitive sleep deprivation has been somewhat highlighted in human studies (Walsh and Poe, 2012), it has not been well characterized in rodent research.

Another important relatively unexplored avenue outside of the studies described in this dissertation pertains to the effects of sleep deprivation over development. Data suggests the effects of sleep deprivation manifest themselves in different ways amongst different age groups. For instance, adolescents appear to be more sensitive to sleep deprivation when it comes to affect and mood compared to other age groups (Talbot et al., 2010). In contrast, the elderly experience a significant reduction in general amounts of sleep (Van Cauter et al., 2000), and are more inclined to experience impaired cognitive functioning than any other age group. These differences in the effects of sleep deprivation may be due to a shift in sleep architecture and/or circadian regulation with age. The contribution of circadian and homeostatic processes in terms of age is not well understood, but is now being explored in humans (Sagaspe et al., 2012). It is possible the mechanics of sleep deprivation disruption on cognitive functioning may be altered as a consequence of age (Porter et al., 2012). This suggests that any future therapeutic

developments would benefit from studies tailored to address differences between these age groups.

The findings reported in this dissertation are in line with the literature demonstrating how even a brief period of sleep loss is detrimental to the hippocampus. It should be noted that while the hippocampus is extremely susceptible to sleep deprivation, other brain regions appear resistant or differently affected. For instance, amygdala function appears to not change or, in many cases, enhances with extended wakefulness. Sleep deprivation has been shown to enhance reactivity in this region as well as other mesolimbic regions associated with response to rewards (Gujar et al., 2011). Apart from this increase in amygdala function, extended wakefulness has been observed to potentiate synapses in cortical regions (Tononi and Cirelli, 2003). This is in contrast to synaptic plasticity being depressed by sleep deprivation in the hippocampus. The contribution of sleep deprivation to these different brain regions needs to be kept in mind when formulating a hypothesis of the role of sleep for the brain. Sleep deprivation does not just inhibit or depress the hippocampus, but also disinhibits particular areas. It is the culmination of this dysregulation induced by sleep deprivation that results in multiple impairments on a behavioral level.

Finally, the underlying mechanics of current therapeutics for sleep loss have not been well characterized. Our lack of mechanistic characterization of current treatment options highlights the lack of understanding concerning how sleep benefits memory and cognition and separately, how sleep deprivation disrupts these processes. Sleep and sleep deprivation are thought to be 2 sides of the same coin and we use sleep deprivation to understand the role of sleep for brain functions. However, it is possible that sleep deprivation may have a separate contribution to the brain outside of an anti-

sleep effect. This suggests that sleep and sleep deprivation may instead be 2 separate dissociable processes that have their own effects on memory.

Author Contributions

Experiments were conceived and designed by Toni-Moi Prince and Dr. Ted Abel. Electrophysiological and behavioral experiments were carried out by Toni-Moi Prince This chapter was written by Toni-Moi Prince with comments and editing by Hannah Schoch, Dr. Jennifer Choi, and Dr. Ted Abel.

APPENDIX

LOSS OF P75^{NTR} HAS COMPLEX EFFECTS ON HIPPOCAMPUS-DEPENDENT MEMORY AND PLASTICITY

Abstract

Neurotrophins are believed to mediate hippocampal function through Trk and $p75^{NTR}$ receptor signaling. Although Trk receptors have been well characterized, the role of $p75^{NTR}$ in memory is controversial and has yet to be clearly defined in comparison to other neurotrophin receptors. Within the limited studies, the findings are contradictory to each other. To elucidate the role of $p75^{NTR}$ in hippocampal function, we examined memory and plasticity in mice deficient in $p75^{NTR}$. Here, we show that loss of $p75^{NTR}$ impaired contextual fear memory, without affecting L-LTP. Although no significant effect of $p75^{NTR}$ deficiency was observed in performance during the object place recognition task, a trend towards enhanced memory existed in this task. We also conducted a preliminary investigation of $p75^{NTR}$ as a potential molecular target of sleep deprivation in the impairment of hippocampal function. An examination of theta burst stimulation induced LTP after sleep deprivation showed that loss of $p75^{NTR}$ did not rescue the reduction in synaptic plasticity. This suggests that while $p75^{NTR}$ may play a role in specific aspects of hippocampus-dependent memory, it may not contribute to the hippocampal deficits induced by sleep deprivation.

Introduction

Neurotrophin signaling is critical for neuronal cell survival, as well as regulation of components required for synaptic plasticity, including neuronal projections, connections, and neurotransmitter release (McAllister et al., 1999; Chao, 2003). Family members include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT3), and neurotrophin 4 (NT4). Neurotrophins have been shown to mediate hippocampus-dependent memory as well as hippocampal long-term potentiation (LTP), which is thought to underlie memory storage (Bramham et al., 1996; Chen et al., 2007; Griffin et al., 2009; Ramos-Languren and Escobar, 2013). The actions of neurotrophins rely on 2 different receptor signaling systems, the Trk receptor tyrosine kinases and the p75 neurotrophin receptor ($p75^{NTR}$) (Chao, 2003). Trk receptor signaling has previously been well-established in the learning and memory literature. In contrast, the role of $p75^{NTR}$, a member of the tumor necrosis factor (TNF) receptor super-family (Bhakar, 1999), is less well characterized. Although its functions are still being discerned, $p75^{NTR}$ has been shown to have an opposing effects to Trk receptors in hippocampus-dependent memory and synaptic plasticity. Loss of $p75^{NTR}$ enhanced fear acquisition memory, whereas loss of TrkB signaling impaired this type memory (Olsen et al., 2013). In addition, the $p75^{NTR}$ knockout mice display enhanced spatial memory in the Barnes maze task (Greferath et al., 2000; Barrett et al., 2010). In regards to hippocampal synaptic plasticity, Trk receptors facilitate LTP and attenuate LTD (Xu et al., 2000; Ikegaya et al., 2002; Minichiello, 2009), while $p75^{NTR}$ facilitates long-term depression (Rösch et al., 2005; Woo et al., 2005; Barrett et al., 2010). Although these findings support opposing roles for the neurotrophin receptors, other work has shown conflicting results produced by loss of $p75^{NTR}$. Peterson and colleagues showed that mice deficient in $p75^{NTR}$ exhibited impaired memory in the inhibitory avoidance task and

the water maze task (Peterson et al., 1999). We want to resolve this discrepancy in the role of $p75^{NTR}$ in memory by examining hippocampus-dependent memory and synaptic plasticity in $p75^{NTR}$ deficient mice.

Separate from the interesting role that $p75^{NTR}$ plays in memory, this receptor has also been observed to affect sleep-wake behavior. $p75^{NTR}$ is a clock controlled gene that is necessary to maintain circadian oscillation (Baeza-Raja et al., 2013). In addition $p75^{NTR}$ is also known to interact with phosphodiesterase (PDE) 4A5, an isoform of the enzyme targeted by sleep deprivation to impair memory and plasticity. This interaction between $p75^{NTR}$ and PDE4A5 results in degradation of cyclic adenosine monophosphate (cAMP) (Sachs and Akassoglou, 2007; Sachs et al., 2007). Reduction in cAMP can impair memory and LTP in the hippocampus (Vecsey et al., 2009). Apart from its interaction with PDE4A5, $p75^{NTR}$ regulates cholinergic neurons in the basal forebrain, a region that plays a large role in sleep-wake behavior. These findings potentially suggest that loss of the receptor could essentially disrupt the balance between sleep and wakefulness as well as alter the effect of sleep deprivation on memory. However, the role of $p75^{NTR}$ in the impact of sleep deprivation on hippocampal function has never been examined.

With these intriguing findings concerning the role of the $p75^{NTR}$ receptor in memory and sleep, we were interested in exploring the effects of loss of this receptor ($p75^{NTR-/-}$) on hippocampus-dependent memory and LTP. We examine long-term memory in these mice because neurotrophin signaling has been observed to play a role in long-term memory but not short-term memory (Callaghan and Kelly, 2013). We specifically explore contextual fear memory and spatial memory because these memories require the hippocampus, a region where these receptor are highly expressed (Hennigan et al., 2007). We also wanted to determine what effect sleep deprivation

would have on these mice, because the $p75^{NTR}$ is a clock gene and known to interact with PDE4A to increase cAMP function. We demonstrate that loss of $p75^{NTR}$ impairs contextual memory, while object place recognition memory is unaltered. Based on our preliminary data, loss of $p75^{NTR}$ function did not affect LTP. Sleep deprivation may still affect L-LTP in these mice, suggesting that $p75^{NTR}$ is not related to impairments induced by sleep deprivation.

Materials and Methods

Mice

Fifty-four adult male mice (2 to 4 months of age) were single housed and kept on a 12h/12h light/dark schedule with lights on at 7:00 AM (ZT 0). The generation of neurotrophin receptor p75 constitutive knockout mice ($p75^{NTR-/-}$) has been previously described (Lee et al., 1992) and were on a C57Bl/6 background (backcrossed for at least 10 generations). WT littermates were used as controls in all experiments. Food and water were available *ad libitum* throughout the experiments. All experiments were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania and were carried out in accordance with all National Institutes of Health guidelines.

Sleep Deprivation

To assess the effects of sleep deprivation (SD) on memory, mice (n = 6) were sleep-deprived for 5 hours using the gentle handling technique involving manual cage tapping, cage shaking, nestlet disturbance, and gentle animal prodding (Ledoux, Sastre, Buda, Luppi, & Jouvet, 1996; Vecsey et al., 2013). For LTP, sleep deprivation was administered immediately before slice collection.

Behavior

Fear Conditioning

Fear conditioning experiments were performed in chambers using the methods previously described (Vecsey et al., 2009). Mice (n = 18) were handled for 3 consecutive days for 1 minute each day. For contextual fear conditioning, mice were placed into the conditioning chamber and received a 2 second 1.5 mA scrambled footshock 2.5 minutes after placement into the chamber. Mice were removed from the chamber after 3 minutes. The test session occurred 24 hours after conditioning during which mice were re-exposed to the context for 5 minutes. Freezing behavior, the complete absence of movement, during the test session indicated the strength of the memory (Fanselow, 1980). The behavior of each mouse was sampled at 5-second intervals and the percentage of those intervals in which the mouse froze was calculated.

Object-place recognition (OPR)

For this task, we used a previously established design that has been shown to be hippocampus dependent (Havekes et al., 2012; Oliveira et al., 2010). Mice (n = 31) were handled for 2 minutes each day, for 6 consecutive days leading up to experimentation. The task was conducted in a grey rectangular box (40 cm x 30 cm x 30 cm) built of polyvinyl chloride plastic. Mice were placed in the empty box for 6 minutes for habituation. Mice were then removed and placed back in the home cage. After 3 minutes, mice were placed in the box with 3 different objects (a 100 ml glass bottle, a white cylinder, and a metallic rectangular tower) for 3 consecutive 6-minute training sessions. Each training session was separated by a 3-minute interval during which the animals were returned to the holding cages. At completion of the training sessions, NSD mice were left undisturbed in their home cages and SD mice were deprived of sleep by

gentle handling. Twenty-four hours following the training session, mice were re-introduced to the spatial context in a single test session. In this session, one of the objects was repositioned (the displaced object: DO), thereby changing the spatial configuration of the objects in the box. Mice were allowed to explore objects for 6 minutes. Exploration was recorded during training and testing on a digital camera for subsequent scoring of time spent exploring objects. Exploration of the objects was defined as the amount of time mice were oriented toward an object with their noses within 1 cm of it, and was measured by an experimenter who was blinded to the experimental history of the mouse.

The change in preference of object exploration between the last training session and test session was used as an index for spatial memory. Preference for an object during the training and test session was determined by calculating the percentage of time spent exploring a particular object versus total time spent exploring all objects. The formula for this measure is as follows: [TEST (DO/(DO + NDO) x 100]- [3rd TRAINING SESSION (DO/(DO+NDO) x 100], where DO = time spent exploring the displaced object and NDO = time spent exploring the non-displaced object. A positive change in percentage exploration of displaced object between test session and the final training session indicates preference for exploring displaced object. This is indicative of an accurate memory for the original locations of objects and that the mice were able to recognize the object's displacement. No change or a negative change in time spent exploring the displaced object in the test session compared to the last training session indicates inability to recognize the new spatial configuration.

Electrophysiology

To assess the effects of SD on hippocampal LTP, electrophysiological recordings were performed as described previously (Vecsey et al., 2009). Mice (n = 14) were either left undisturbed in their home cages (NSD control group), or subjected to 5 hours of SD. Immediately after this manipulation, SD and time-matched NSD mice were sacrificed by cervical dislocation, and their hippocampi were dissected rapidly in iced oxygenated artificial cerebrospinal fluid (aCSF; 124 mM NaCl, 4.4 mM KCl, 1.3 mM $MgSO_4*7H_2O$, 1 $NaH_2PO_4*H_2O$, 26.2 mM $NaHCO_3$, 2.5 mM$CaCl_2*2H_2O$, 10 mM glucose). 400 μm thick transverse hippocampal slices were prepared using a Stoelting tissue chopper (Stoelting Co. Wood Dale, IL) and placed in an interface chamber and continuously perfused with oxygenated aCSF while they equilibrated for at least 1.5 hours at 28.0ºC before starting electrophysiological recordings. Single pathway recordings were made using a single bipolar stimulating electrode (A-M Systems; 0.002 inches diameter nichrome wire) placed in the stratum radiatum of the CA1 subfield to elicit action potentials in the axons of CA3 pyramidal neurons. An aCSF-filled glass microelectrode (A-M Systems; 1.5 mm x 0.85 mm) with a resistance between 0.5 and 3 MΩ placed in the stratum radiatum region of CA1 was used to record the resulting field excitatory post-synaptic potential (fEPSP). Data were acquired and analyzed using Clampex 8.2 software (Molecular Devices, Palo Alto, CA). Peak fEPSP amplitude was required to be at least 5 mV, and stimulus intensity was set to produce 40% of the maximal response. Test stimulation occurred once every minute. A 20-minute baseline period was recorded in each experiment prior to stimulation. To examine late-phase LTP (L-LTP), we used theta burst stimulation, which consists of 40-ms duration, 100-Hz bursts delivered at 5Hz for 3 seconds (15 bursts of 4 pulses per burst, for a total of 60 pulses). Induction of this form of LTP depends on the NMDA receptor, and its maintenance depends on cAMP (Nguyen and

Kandel, 1997). Recordings continued for at least 1 hour after LTP induction. Initial fEPSP slopes were normalized against the average of the 20 baseline traces.

Statistical Analysis

Two-way ANOVA was used to analyze spatial preference in the OPR task, with genotype and objects as factors and the delta percent exploration as the dependent variable. A student's t-test was used to analyze the average of the last 20 minutes of fEPSP slopes from potentiated hippocampal slices from the WT group and the $p75^{NTR-/-}$ group. A student's t-test was used to analyze the average freezing during the test session of CFC of the WT group and $p75^{NTR-/-}$. Tukey's multiple comparison was selected for post hoc analyses for OPR. All statistical analyses were performed using SAS 9.3 software (SAS Institute Inc., Cary, NC). A probability level of $p < 0.05$ was considered significant. Data are presented as mean ± SEM.

Results

Loss of $p75^{NTR}$ function reduces freezing in contextual fear conditioning.

Loss of $p75^{NTR}$ has previously been shown to enhance fear memory in the inhibitory avoidance task (Olsen et al., 2013). We wanted to examine the effects of loss of $p75^{NTR}$ in another fear memory task, contextual fear conditioning. Student's t-test revealed that freezing in $p75^{NTR-/-}$ mice during the test session was reduced compared to the WT control group (**Fig A.1.:** $t_{(15)} = 3.24$, $p < 0.05$; WT = 45.7 ± 5.66%, $p75^{NTR-/-}$ = 23.4 ± 5.51%). In contrast to previous findings, this result suggests that loss of $p75^{NTR}$ impairs contextual memory.

Loss of $p75^{NTR}$ function may affect memory in the OPR task.

$p75^{NTR-/-}$ mice have previously displayed enhanced spatial memory in the Barnes maze task, which researchers hypothesized to be due to increased cholinergic input (Barrett et al., 2010). We wanted to explore long-term memory in another spatial memory task that requires less training trials. We examined the impact of loss of $p75^{NTR}$ function on long term memory in the OPR task. Change in exploration for the DO and NDO was compared between $p75^{NTR-/-}$ group and the WT control group. A two-way ANOVA revealed that there was no effect of genotype on exploration of objects (**Fig A.2.:** $F_{(1,40)} = 0.26$, $p = 0.61$). There was a significant main effect of object type (**Fig A.2.:** $F_{(1,40)} = 29.16$, $p < 0.05$. Although the interaction between genotype and object type did not reach significance, a trend for enhancement in $p75^{NTR-/-}$ exploration of the DO was observed (**Fig A.2.:** $F_{(1,40)} = 2.92$, $p = 0.09$). A trend existed for increased percent exploration of the DO over the period of the last training session to the test session for the WT group (DO = 9.77 ± 3.98%; NDO = -4.52 ± 2.82%). There was a significant increase in percent exploration for the DO compared to the NDO for the $p75^{NTR-/-}$ group (DO = 18.35 ± 5.74%; NDO = -9.18 ± 3.01%). A trend towards enhancement in $p75^{NTR-/-}$ mice suggests that reduced $p75^{NTR}$ receptor signaling enhances spatial memory.

Preliminary data suggest that loss of $p75^{NTR}$ does not prevent sleep deprivation induced impairment seen in theta burst stimulation (TBS) late-phase LTP.

We examined long-lasting LTP induced by TBS. This type of stimulation induces LTP that requires protein synthesis and cAMP signaling. cAMP is known to be disrupted by this receptor's interaction with PDE4A5, an enzyme that increases activity as a result of sleep deprivation. We examined the effect of sleep loss on TBS-LTP in slices from WT and $p75^{NTR-/-}$ groups (**Fig. A.3. A** and **A.3. B**). Sleep deprivation appeared to reduce LTP compared to the LTP exhibited in slices from non-sleep deprived (NSD) controls

(WT NSD = 179 ± 46.7%, WT SD = 133 ± 54.4%, $p75^{NTR-/-}$ NSD = 214 ± 35.2%, $p75^{NTR-/-}$ SD = 141% ± 30.4%). Loss of $p75^{NTR}$ does not appear to rescue sleep deprivation-induced impairment of late-phase LTP induced by TBS. We also examined if $p75^{NTR}$ deficiency alters TBS-LTP. Loss of $p75^{NTR}$ may slightly enhance L-LTP (**Fig. A.3. C**).

Discussion

Our study investigated whether $p75^{NTR}$ plays a role in memory consolidation, and whether this receptor is a mediator in the impairment of hippocampal synaptic plasticity after sleep deprivation. We found that loss of $p75^{NTR}$ impaired memory in CFC (**Fig. A.1.**). $p75^{NTR}$ related signaling is not a requirement for spatial memory as suggested by performance in the OPR task (**Fig. A.2.**). There was a trend towards impaired LTP as a result of sleep deprivation (**Fig. A.3.**). Sleep deprivation appeared to impair LTP induced by TBS in WT and $p75^{NTR-/-}$ mice (**Fig. A.3.**). Thus, $p75^{NTR}$ contributes to memory consolidation. However, $p75^{NTR}$ may not be a mediator of sleep deprivation-induced impairment in hippocampal functioning.

We unexpectedly found that loss of $p75^{NTR}$ impaired memory in contextual fear conditioning (**Fig. A.1.**). This was unexpected because $p75^{NTR}$ is considered to have an opposing effect to the other neurotrophin receptors (Lu et al., 2005). Knowing that Trk receptor signaling has been shown to enhance fear memory (Choi et al., 2012; Zeng et al., 2012; Olsen et al., 2013) led to our initial hypothesis that loss of $p75^{NTR}$ would enhance memory in CFC. One possible explanation for this surprising result is the loss of interaction between $p75^{NTR}$ with the TrkB receptors. These receptors often work in conjunction to modulate signal transduction by refining ligand fidelity. This increase in ligand fidelity lead to increased affinity for TrkB to bind to BDNF (Chao and Hempstead, 1995; Greene and Kaplan, 1995; Bibel et al., 1999; Roux and Barker, 2002; Chao, 2003;

Lad et al., 2003). On the other hand, we do not see this effect in other hippocampus-dependent memory tasks (**Fig. A.2.**) suggesting that there is more to the observed CFC memory deficit in $p75^{NTR-/-}$ mice.

Another plausible explanation for our observation of that $p75^{NTR-/-}$ mice demonstrate impaired memory in CFC is that loss of $p75^{NTR}$ is associated with significant neuronal reduction throughout the central nervous system which could affect fear memory along with other functions (Peterson et al., 1999). Although other work has shown enhanced memory linked to increased cholinergic signaling in $p75^{NTR-/-}$ (Barrett et al., 2010), Peterson and colleagues observed reduced cholinergic neurons in the basal forebrain (Peterson et al., 1999). This discrepancy in findings has not been reconciled, and suggests that further study is imperative to determine the role of this receptor in regulating cholinergic neurons in the basal forebrain. Although the differences between the findings of these two studies have yet to be resolved, it is likely that these $p75^{NTR-/-}$ mice have global deficits resulting in impaired performance in particular tasks. Previous work on these mice showed that loss of $p75^{NTR}$ resulted in impaired hippocampus-dependent memory in the inhibitory avoidance task, another fear memory task, and the water maze task, another spatial memory task (Peterson et al., 1999). These mice also displayed impaired motor activity and deficits in habituation tasks (Peterson et al., 1999). Separately, generation of these particular knockout mice also results in impaired innervations of peripheral sensory neurons as well as decreased sensitivity to NGF (Lee et al., 1994a, 1994b). Impaired innervation of peripheral sensory neurons would affect how animals perform in tasks that rely on sensation or mobility, such as any shock-related task or swimming task. This may explain why we observe a deficit in CFC, but not in OPR. More studies are required to assess the precise role of this receptor in learning and memory.

Our LTP studies are only preliminary, but we did not observe impairments in TBS-LTP in $p75^{NTR-/-}$ mice (**Fig. A.3.**). Originally, examination of theta burst-induced L-LTP (TBS-LTP) was chosen because this form is regulated by neurotrophin signaling as well as by cAMP. We observed a trend to reduced TBS-LTP after sleep deprivation similar to previous studies (Vecsey et al., 2009). Although we cannot definitively conclude anything from this LTP data, we can discuss our rationale for choosing to examine this experiment. Reduction in cAMP by the PDE4A5 results in impaired LTP. Sleep deprivation is a driving force for these molecular changes, which results in impaired LTP (Vecsey et al., 2009). $p75^{NTR}$ interacts with PDE4A5 to degrade cAMP in a PKA-dependent fashion (Sachs et al., 2007).Due to the interaction between $p75^{NTR}$ and PDE4A5 to reduce cAMP, we predicted that $p75^{NTR-/-}$ mice would be resistant to the effects of sleep deprivation on LTP. However, after more examination into the mechanism of how sleep deprivation seems to affect PDE4A5 regulation and how $p75^{NTR}$ interacts with PDE4A5, involvement of $p75^{NTR}$ in the effects of sleep deprivation through the PDE4A5 pathway seems unlikely. PDE4 inhibition by rolipram works by interacting with the catalytic domain (Sachs and Akassoglou, 2007), suggesting that sleep deprivation exerts effects by disrupting the catalytic domain. $p75^{NTR}$, in contrast, interacts with the C-terminal of PDE4A5 and therefore sleep deprivation may not alter this interaction (Sachs and Akassoglou, 2007). Although $p75^{NTR}$ does not interact with PDE4A5 in the same region as rolipram, the process by which sleep deprivation disrupts hippocampal function is relatively unknown. $p75^{NTR}$ could play an indirect role in enhancing cognitive susceptibility to sleep loss by interacting with PDE4A5. This requires further examination of whether the interaction of these proteins is regulated by sleep deprivation.

The questions pertaining to the role of $p75^{NTR}$ in memory and in the impact sleep deprivation on hippocampal function are interesting. We used a constitutive knockout to determine the sufficiency of $p75^{NTR}$ in functions related to the hippocampus. The benefit of using a knockout is that it provides information regarding gene function *in vivo*. However, due to the existing translational models, these questions remain unresolved. The $p75^{NTR-/-}$ mouse model used in our study, also known as $p75^{NTR\Delta Exon3}$, appear to have global deficits. This hinders behavioral assessments pertaining to learning and memory because global deficits could mask the effects of loss of $p75^{NTR}$ on the hippocampus, our region of interest. In addition, these mice may express an alternate spliced isoform that may be able to support signaling activity (von Schack et al., 2001), and are therefore an inadequate model to determine the role of the receptor. The other option, $p75^{NTR\Delta Exon4}$, is not optimal either. Forty percent die during the late fetal or early postnatal period. The surviving mice display growth retardation, and have abnormal reflexes, as well as impaired motility (von Schack et al., 2001). These conditions are not ideal for examining performance in learning and memory tasks, and as a consequence, interpretation of phenotypes is flawed. Development of conditional mutants is needed to assess the role of this receptor in specific tissue. Generation of conditional animals have been created recently and represent an alternative approach (Bogenmann et al., 2011). This, along with pharmacological suppression of $p75^{NTR}$, may provide a novel avenue to more critically examine the effects of this receptor in learning and memory.

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Author Contributions

Experiments were conceived and designed by Toni-Moi Prince and Dr. Ted Abel. Electrophysiological and behavioral experiments were carried out by Toni-Moi Prince This chapter was written by Toni-Moi Prince with comments and editing by Dr. Jennifer Choi and Dr. Ted Abel.

FIGURE A.1.

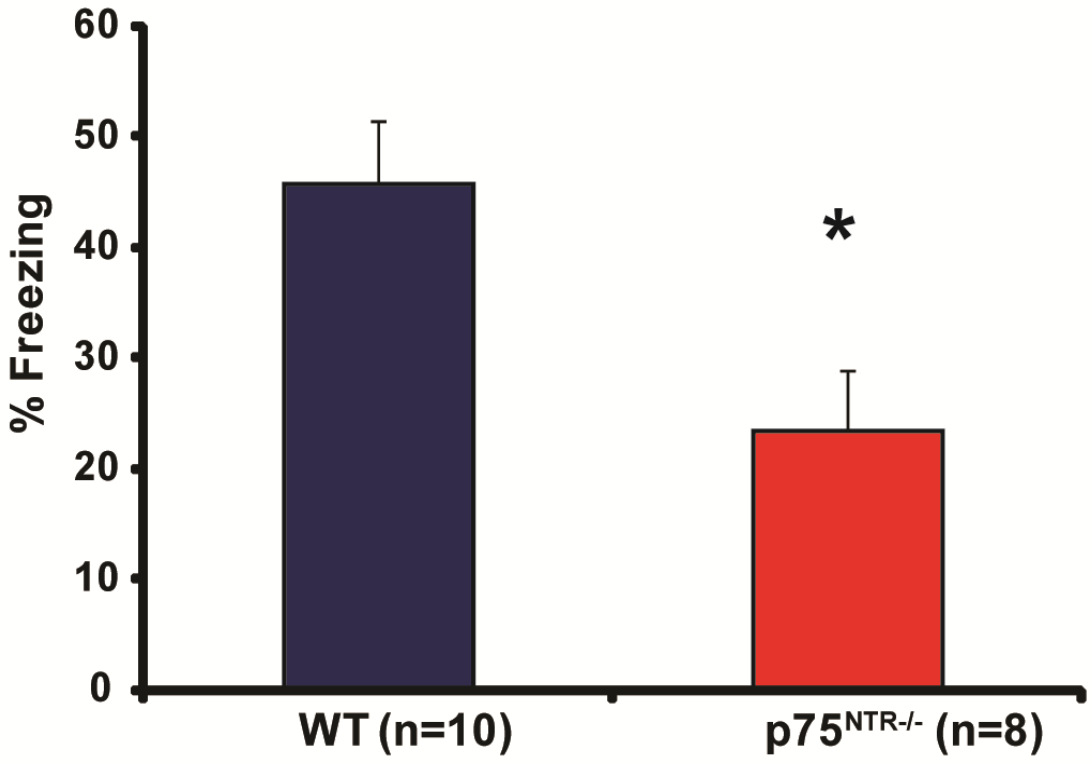

Figure A.1. *Loss of p75^{NTR} impairs contextual fear memory.*

Mean delta freezing during the test session was significantly lower in the p75$^{NTR-/-}$ mice (n = 8) compared to their WT littermates (n = 10) ($t_{(15)} = 3.24$; $p < 0.05$). Error bars indicate ± SEM.

FIGURE A.2.

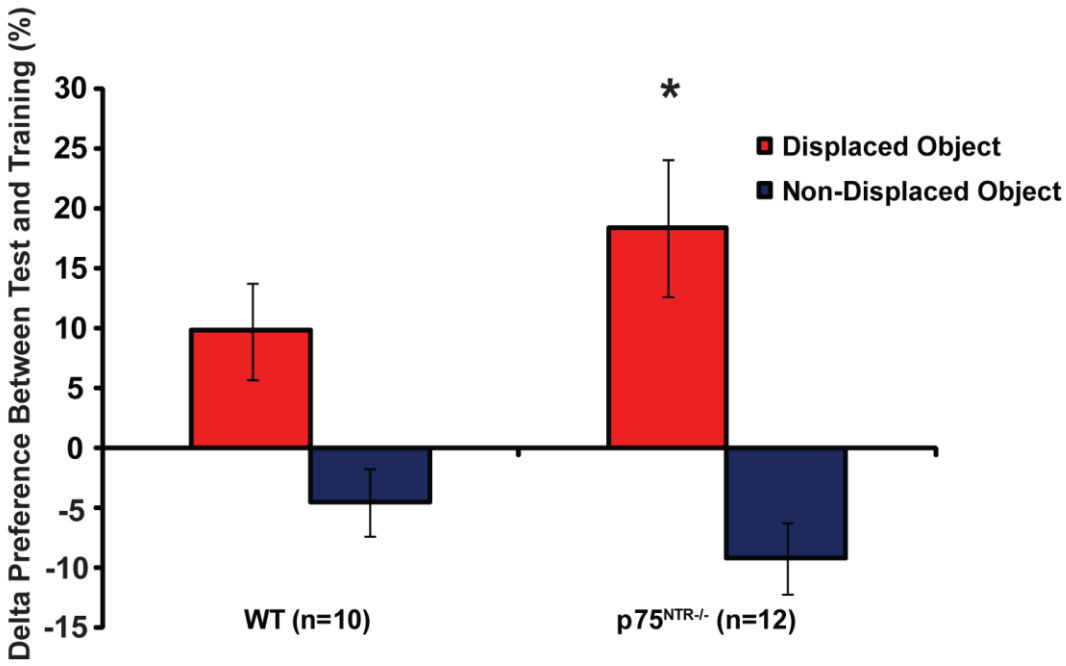

Figure A.2. *Loss of p75^{NTR} does not affect spatial memory in the OPR task.*

Mean delta percentage exploration of the DO increased compared to exploration of the NDO for the p75$^{NTR-/-}$ group (n = 12) ($F_{(1,40)}$ = 29.16, $p < 0.05$: $t_{(40)}$ = 5.27 $p < 0.05$). Mean delta percentage exploration of the DO was not significantly different than NDO for the WT group (n = 10) but approached significance $F_{(1,40)}$ = 29.16, $p < 0.05$: $t_{(40)}$ = 2.50; p = 0.07). Mean exploration of the DO did not differ between WT and p75$^{NTR-/-}$ groups ($F_{(1,40)}$ = 2.92; p = 09: $t_{(40)}$ = -1.57, p = 0.41). Mean exploration of the NDO did not differ between WT and p75$^{NTR-/-}$ groups ($F_{(1,40)}$ = 2.92; p = 09: $t_{(40)}$ = 0.85, p = 0.83). Error bars indicate ± SEM.

FIGURE A.3.

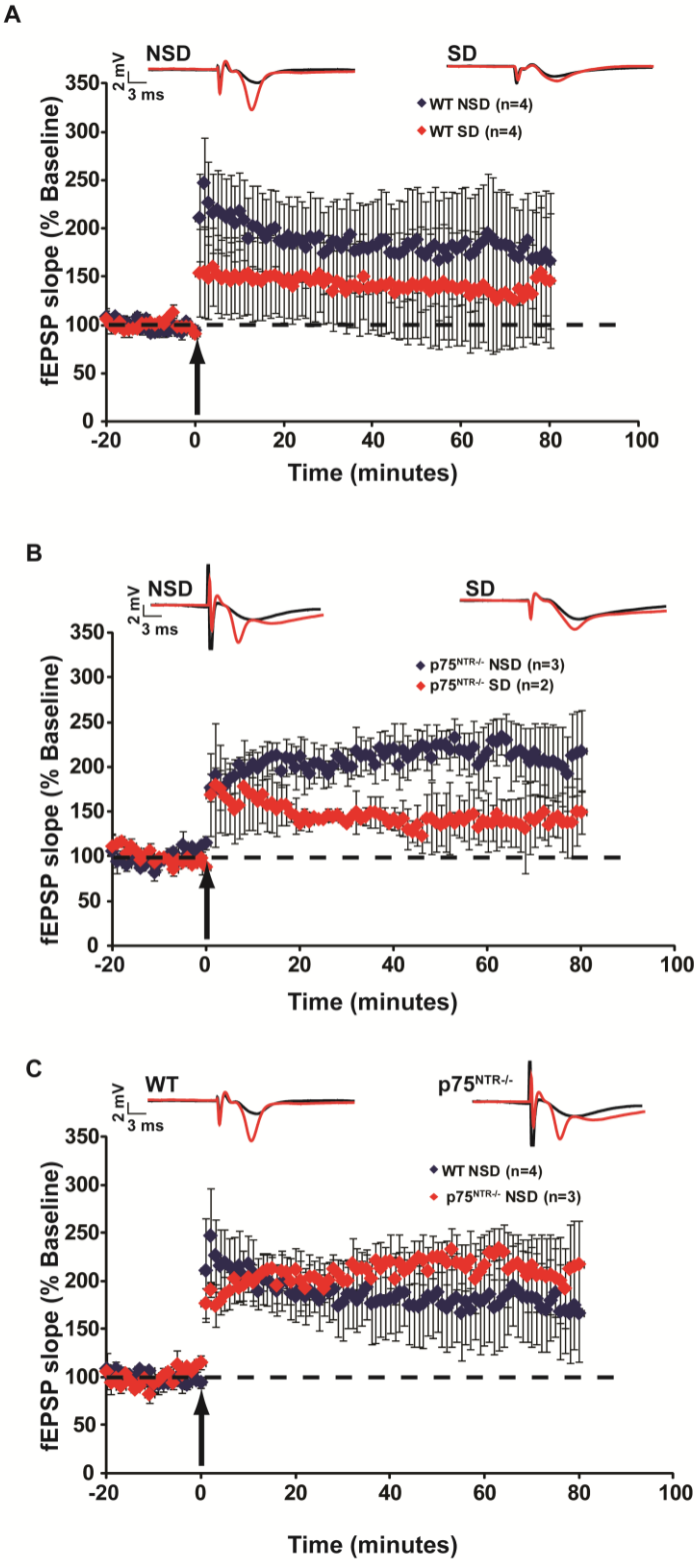
A
NSD
SD
2 mV
3 ms
WT NSD (n=4)
WT SD (n=4)
fEPSP slope (% Baseline)
Time (minutes)
B
NSD
SD
2 mV
3 ms
p75NTR-/- NSD (n=3)
p75NTR-/- SD (n=2)
fEPSP slope (% Baseline)
Time (minutes)
C
WT
p75NTR-/-
2 mV
3 ms
WT NSD (n=4)
p75NTR-/- NSD (n=3)
fEPSP slope (% Baseline)
Time (minutes)

Figure A.3. *Preliminary Results: Loss of p75^{NTR} does not appear to alter the effects of sleep deprivation on LTP induced by theta burst stimulation.*

(A) There is a trend toward reduced L-LTP induced by TBS as a result of sleep deprivation in WT animals. (NSD, n = 4; SD, n = 4). (B) There is a trend toward reduced L-LTP induced by TBS as a result of sleep deprivation in p75$^{NTR-/-}$ (NSD, n = 3; SD, n = 2). (C) L-LTP induced by TBS is not altered by p75$^{NTR-/-}$ (WT, n = 4; p75$^{NTR-/-}$, n = 3). Error bars indicate ± SEM.

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